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Characterization of Rapidly Adhering Amniotic Fluid Cells by Combined Immunofluorescence and Phagocytosis Assays

K. Polgár,* R. Ádány,† G. Ábel,‡ J. Kappelmayer,† L. Muszbek,† and Z. Papp*

*Department of Obstetrics and Gynecology, †Department of Clinical Chemistry, and ‡Institute of Pathophysiology, University Medical School of Debrecen, Debrecen, Hungary

Summary

Culture of human amniotic-fluid cells from cases of fetal neural tube defects produces a population of rapidly adhering cells that were initially thought to be macrophages and later interpreted to be of neural origin. In this study double and triple labeling systems for the simultaneous detection of glial and macrophage differentiation marker antigens have been used to demonstrate that rapidly adhering cells cannot be considered a homogeneous population but instead represent two distinct cell types. One of these cell populations is of glial origin and shows specific staining for glial fibrillary acidic protein, while the other population is monocyte-derived macrophages which express marker antigens recognized by Leu M3, KiM7, and Dako antimacrophage monoclonal antibodies.

Introduction

Both determination of alpha-fetoprotein (AFP) levels and ultrasonic methods have been widely used in the prenatal diagnosis of neural tube defects (NTDs). Because neither amniotic-fluid AFP estimation or ultrasonography is entirely reliable in the diagnosis or exclusion of neural tube closure defects (Chapman 1982; Mizejewski and Risemberg 1985), cytological diagnostic methods have been developed. In cases of fetal NTDs a large number of macrophages are present in amniotic fluid. Some of these cells can be recognized on the basis of morphological properties by direct microscopical examination of cytologic smears (Papp and Bell 1979; Chapman 1982; Papp et al. 1982). More recently, simple color tests based on the pinocytotic properties of the pathognomonic cells were introduced, and these cells are easily identified as "red cells" by light microscopy (Polgár et al. 1984, 1985).

Although rapidly adhering (RA) cells, which settle in less than 24 h during cultivation of amniotic-fluid cells, have been identified as macrophages (Sutherland et al. 1973), contradictory results have also been published. It has been suggested that most of the RA cells are of neural origin (Gosden and Brock 1977), but some studies have shown that RA cells are not homogeneous and that the proportion of neural cells present is variable (Aula et al. 1980; Cremer et al. 1981; Medina-Gomez and Bride 1986).

The present study of amniotic fluid was designed to (1) immunomorphologically characterize RA cells appearing in combination with NTDs and (2) identify the cell population involved in phagocytosis.

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Address for correspondence and reprints: Dr. Katalin Polgár, Department of Obstetrics and Gynecology, University Medical School of Debrecen, P.O. Box 37, H-4012 Debrecen, Hungary.

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Material and Methods

Case Studied

Amniotic-fluid samples were obtained by transabdominal amniocentesis from 11 patients of the Genetic Counseling Service of the Department of Obstetrics and Gynecology, University School of Medicine, Debrecen, Hungary between May 10, 1985, and April 21, 1988. Amniocenteses were carried out for different indications: normal or suspicious ultrasound findings, high maternal serum AFP, etc. Pregnancies were terminated by induced abortion, and the following diagnoses were confirmed by embryopathological investigations: seven cases of anencephaly (two of these fetuses also had spina bifida) and four cases of spina bifida.

Samples obtained by amniocentesis were divided into two parts. One part was used for making cytospin preparations, and the other was used for short-term cultivation of amniotic-fluid cells to obtain RA cell monolayers.

Cytospin Preparations

Amniotic-fluid cell preparations were made by cytospin 2 centrifugation at 800 rpm for 5 min (Shandon Southern Products, Runcorn, Cheshire).

Cell Culture

To obtain primary adherent cell monolayers, amniotic fluid was cultured in flaskette tissue culture chambers containing Chang C medium (HANA Biologics, Inc., Alameda), by incubation at 37°C in a humidified atmosphere of 5% CO_2 in air. After 20–24 h the supernatants, which contained mainly unattached amniotic-fluid cells and red blood cells, were removed. Slides covered by RA cell monolayers were processed for microscopy.

Fixation

Both air-dried cytospin preparations and primary adherent monolayers were washed in PBS, pH 7.4 and were fixed with absolute acetone at room temperature for 10 min.

Proteins

Human complement component C3 was purchased from Cordis Laboratories (Miami). C3b was prepared by trypsin digestion of C3 according to a method described elsewhere (Ogle et al. 1985). Human IgG was purchased from Calbiochem (Lucern).

Coupling of Proteins to Microspheres

Covalent coupling of proteins (C3b or IgG) to fluorescent carboxylated microbeads ("Fluoresbrite," 0.88 µm diameter; Polysciences, Warrington, PA) was performed according to a method described elsewhere (Ogle et al. 1985).

Phagocytosis Assay Procedure

Details of the procedure are described elsewhere (Abel et al., in press). In brief, phagocytosis of fluorescent microbeads was assayed as follows: 100 µl of the desired type of fluorescent microspheres (10^9 particles/ml) was added to 1 ml amniotic-fluid cells suspended in Hanks' balanced salt solution (HBSS) at 10^6 cells/ml. Thus, the particle:cell ratio was 1,000:1. These mixtures were incubated for 60 min at 37°C, layered on top of 3 ml cold PBS containing 6% BSA, and centrifuged at 400 *g* for 5 min (Ogle et al. 1985). The supernatant fluid was carefully aspirated, and the pellet was resuspended in 1 ml HBSS containing 1% BSA and 0.2% sodium azide. This cell suspension was used for cytocentrifuge preparations and fluorescence microscopy.

Triple Labeling Systems (double immunofluorescence + cytochemical reaction)

On acetone-fixed cytospin preparations and primary cell cultures, nonspecific IgG binding was blocked by incubation for 10 min with 5% normal goat serum in PBS. Glial fibrillary acidic protein (GFAP) was detected by indirect immunofluorescence. Samples were incubated with the first antiserum (according to directions supplied by the vendor of the GFAP assay kit [Ortho Diagnostic Systems Inc., Raritan, NJ]) for 1 h and then with a 1:40 dilution of fluoresceinated porcine anti-rabbit IgG (Dakopatts a/s, Glostrup, Denmark) for 45 min.

This reaction was followed with one of the following three immunoreactions: (1) detection of Leu M3, a surface marker of monocyte/macrophage cell line (Dimitriu-Bona et al. 1983), stained by 45 min incubation with a 1:5 dilution of monoclonal antibody conjugated with phycoerythrein (Becton Dickinson, Sunnyvale, CA, USA); (2) visualization of a macrophage antigen by Dako-antimacrophage monoclonal antibody (Dakopatts a/s, Glostrup, Denmark) by incubation for 45 min with a 1:10 dilution of the immunoreagent; (3) immunostaining with KiM7 monoclonal antibody, which recognizes an antigen of M_r 29,000 present in the phagocytosing compartment of the monocyte/macrophage cell lineage (Kreipe et al. 1987). The antibody KiM7 (Behringwerke, Marburg, West Germany) was used in a 45-min incubation at a 1:10 dilution. For reactions 2 and 3, horse biotinylated anti-mouse IgG (Vector Labs, Burlingame, CA) was used as second antibody (1:250 dilution, 30-min incubation), and the biotinylated antibody was visualized by incubation for 45 min with a 1:40 dilution of Texas Red streptavidin (Amersham, Arlington Heights, IL). Cytochemical reaction for α-napthyl-acetate esterase (ANAE), a marker enzyme for macrophages (Mueller et al. 1975), was carried out according to directions supplied by the vendor of the assay kit (Sigma, St. Louis).

Combination of Immunofluorescence Reactions with Phagocytosis Assays

Cytospin preparations made from cell suspensions incubated with fluorescent microbeads were fixed in 3.5% paraformaldehyde fixative for 45 min at room

temperature and were stained for each of the antigens described above. The monoclonal reactions were carried out as mentioned above. The binding of primary antibody to GFAP was detected by biotinylated anti-rabbit IgG (1:250 dilution, 30 min; Vector Labs) and Texas Red streptavidin (1:40 dilution, 30 min; Amersham). These preparations were also stained for ANAE.

On control slides, antiserum against GFAP was replaced with nonimmune rabbit serum, while monoclonal antibodies were replaced with control mouse IgG from tumor-bearing BALB/c mice (Becton Dickinson, Sunnyvale, CA). Cytospin-prepared slides were covered with 50% glycerol in PBS and examined with a microscope equipped with an epifluorescence condenser containing selective filters for fluorescein and Texas Red/phycoerythrin. The excitation maximum/emission maximum of fluorescein is 495/525 nm; that of Texas Red is 595/620 nm; and that of phycoerythrin is 496/576 nm. Fluorescence photomicrographs were taken on Scotch 1000 ASA color slide film. Fields photographed under fluorescent illumination were also photographed under bright field illumination.

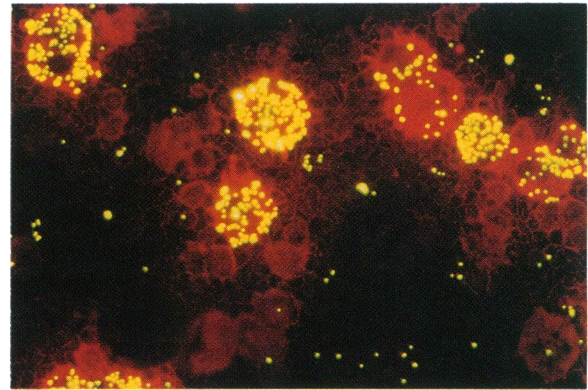


Figure 1 Reaction with Dakop-antimacrophage monoclonal antibody developed by biotinylated second antibody and Texas-Red streptavidin system on amniotic-fluid cell preparation after phagocytosis of fluoresceinated latex microspheres. (× 240).

Results

Cells in amniotic fluid from pregnancies with open NTDs show characteristic morphological features. Many cells showed positive staining for GFAP, and many cells were labeled for macrophage differentiation marker antigens recognized by Leu M3, KiM7, or Dako-antimacrophage monoclonal antibodies. On RA cell monolayers few cells (1%–2%) failed to show labeling for either GFAP or macrophage markers. In double immunofluorescence assays, no doubly labeled cells were observed in cytospin preparations; GFAP$^+$ cells were not observed to express macrophage differentiation marker antigens, and macrophages did not stain for GFAP.

GFAP$^+$ cells varied both in size and in morphological appearance. Such cells were found both as relatively small, round cells of about 15 μm diameter and as larger pseudopodial cells of about 50 μm diameter. Cells showing macrophage-specific reactions mainly appeared as round mononuclear cells, but they varied markedly in apparent size.

Fluorescent latex microspheres were taken up only by cells recognized by Leu M3, KiM7, and Dako-antimacrophage monoclonal antibodies (fig. 1). All cells positive for macrophage differentiation antigens ingested IgG-coated latex microbeads, and cells which phagocytosed fluorescent particles invariably labeled for macrophage antigens. GFAP$^+$ cells did not demonstrate phagocytic activity (fig. 2). All cells expressing macrophage differentiation antigens were also observed to be positive for ANAE.

In primary cultures a significant proportion (20%–40%) of cells showed cytoplasmic fibrillar staining for GFAP. No double reactions with glial cell–specific and macrophage-specific reagents were observed (fig. 3).

In triple labeling experiments complete codistribution could be demonstrated for macrophage markers (KiM7 and Dako-antimacrophage) and ANAE, while GFAP$^+$ cells were consistently negative for these macrophage markers (fig. 4).

Among cells negative for the above-mentioned marker

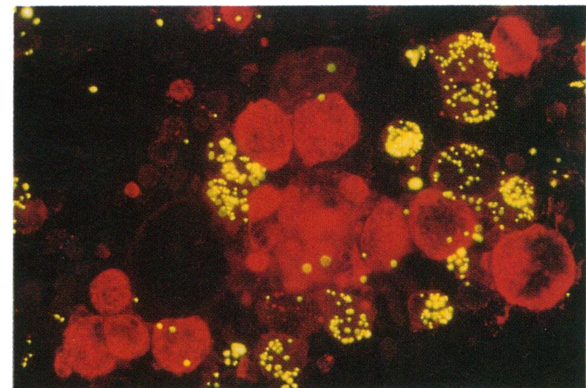


Figure 2 Immunofluorescent labeling for GFAP (in red) in combination with FITC-latex microbead phagocytosis on amniotic fluid cell preparation. GFAP-positive cells do not ingest latex particles. (× 240).

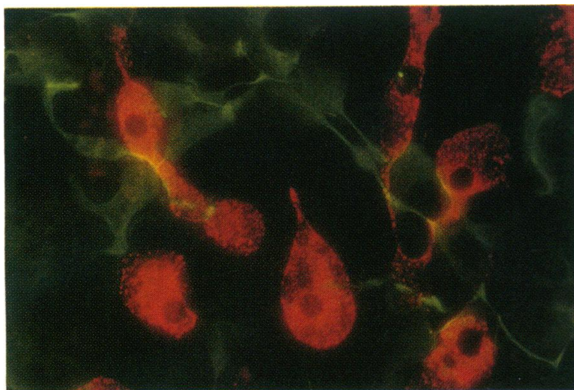


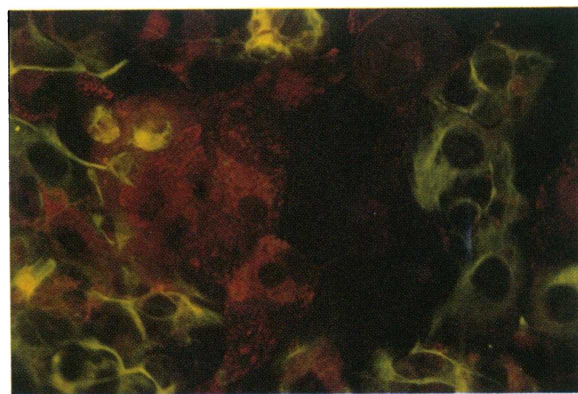
Figure 3 Double immunofluorescent reaction for GFAP (in green) and with Dako-antimacrophage monoclonal antibody (in red) on rapidly adhering amniotic-fluid cells. (× 240).

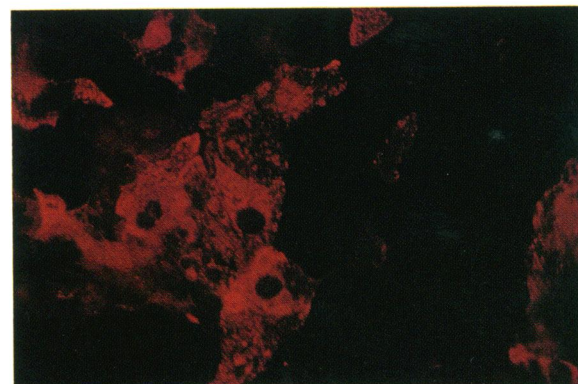
antigens, epithelial cells and red blood cells were easily recognizable on the basis of their characteristic morphology, but a significant number of other cell types could not be identified by morphological features alone.

Discussion

Controversy exists over the origin of mononuclear cells that are present in amniotic fluid in cases of fetal NTDs. Glial origin of the abnormal cells in the amniotic fluid has been suggested (Aula et al. 1980; Sarkar et al. 1980; Cremer et al. 1981; von Koskull et al. 1981; Medina-Gomez and Bride 1986; Polgár et al. 1987). However, others have found no evidence for differences among the abnormal cells with the use of a specific antibody to GFAP (Chapman 1982).

We reported earlier, from results obtained by the peroxidase-antiperoxidase (PAP) method, the possible neural (glial) origin of a part of amniotic-fluid pathognomonic cells (Polgár et al. 1987). Recently, we tried to find and apply specific markers diagnostic of mononuclear phagocytes (van Furth 1986; Polgár et al. 1988*c*). We could demonstrate (*a*) strong endogenous peroxidase and nonspecific esterase activity in these cells on cytologic smears (Polgár et al. 1987) and (*b*) positive immunostaining for both MO1 (63D3 human monocyte 1) and MO2 (61D3 human monocyte 2) antigens (Polgár et al. 1988*c*). We could also demonstrate the presence of functional markers such as Fc and C3b receptor-mediated phagocytosis and chemiluminescence (Polgár et al. 1988*a*). Further evidence of the monocytic origin of many of these cells has been presented elsewhere (Polgár et al. 1984, 1988*b*).





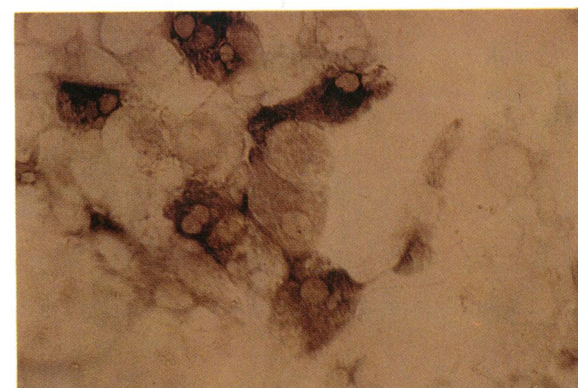


Figure 4 Triple labeling for GFAP (*top*), KiM7 (*middle*), and ANAE (*bottom*) GFAP-positive and KiM7-positive cells are clearly separated from each other. Only KiM7-positive cells show enzyme cytochemical labeling for ANAE. (× 240).

GFAP has been identified elsewhere as a component of astroglial cells, reactive microglia, and tumor-forming astrocytes (Bignami and Dahl 1974; Miyake et al. 1988). GFAP is also expressed by myelin-forming oligodendrocytes in their early development, indicating that the relationship between astroglial and oligodendroglial cells is closer than previously has been believed (Choi

and Kim 1984). It is suggested that in postnatal development two distinct glioblast lines develop, one developing into astrocytes and the other into oligodendrocytes. The primary glioblast is a bipotential glial progenitor cell (Bunge and Waksman 1985). Animal experiments demonstrated that both astroglial cells (Noske et al. 1982) and microglia (Brierley and Brown 1982), rather than oligodendrocytes (Al-Ali and Robinson 1984), become active forms and may act as phagocytes in certain situations, such as brain injury or removal to cell culture. Therefore, pathological development of neural tubes and the activation and release of glial cells or brain macrophages may be caused by direct contact between neural tissue and amniotic fluid (Polgár et al. 1987).

Brain macrophages and microglial cells are considered to be the most important phagocytic elements within the central nervous system (Esiri and Booss 1984; Gosden and Brock 1977). It is interesting that microglial cells, the only type of brain phagocytes which express Fc receptor activity similar to that of amniotic-fluid macrophages (Sutherland et al. 1973), do not share GFAP positivity (Raff et al. 1979). Microglial cells showed positive staining with nonspecific esterase stains, as do monocytes and macrophages (Esiri and Booss 1984). Later it was found that microglial cells lack macrophage markers (Wood et al. 1979). These cells are characterized by 100% MO1 and 10% MO2 reactivity (Franklin et al. 1986). It is interesting that they do not share lysozyme positivity (Esiri and Booss 1984). The controversy over the relationship between macrophage and microglia, as well as over the nature and functional role of microglia in nervous tissue, has been renewed (Choi and Kim 1984).

According to previous work, microglial cells of the brain are included in the mononuclear phagocyte system (van Furth 1986). Many of these macrophages clearly originate from blood-borne monocytes and invade the central nervous system parenchyme via the vascular route (Murabe and Sano 1983). The migration of hematogenous cells into neural tissue during embryogenesis is a common phenomenon in human embryos and fetuses (Choi and Kim 1984). Other phagocytes may arise from microglia resident in the brain which are mobilized after injury (Brierley and Brown 1982). Investigators have suggested that microglia may transform into phagocytes (Oechmichen 1975). Other studies found no evidence that monocytes or macrophages differentiate into microglia or that brain macrophages are derived from microglia (Gordon 1986; Schelper and Adrian 1986). New data call into question the validity of any hypothesis that suggests a common origin for microglia and macrophages. Studies are underway to identify the origin and function of true microglia.

Several studies suggest, however, that astrocytes are able to phagocytose in vivo (Noske et al. 1982). We were not able to demonstrate phagocytic properties among either noncultured or cultured GFAP$^+$ amniotic-fluid cells. After we carried out C3b-coated latex-bead treatment, we could not visualize beads actually internalized by pathognomonic cells in suspension or in primary culture. This may be due to the fact that phagocytosis by astroglial cells is influenced by the stage of differentiation. During embryonic development of the brain, cells of various lineages may have similar morphologies, making it difficult to distinguish between them.

Considering that the microglial cells are of mesodermal hematogenous origin, that macrophages have a monoblastic origin, and that both of them take part in the mononuclear phagocyte system, we conclude that the GFAP-negative pathognomonic cells in cases of fetal NTD are of hematogenous monocytic origin.

The main conclusion to be drawn from the results of the present study is that the mononuclear cell population present in amniotic fluid in cases of open NTDs is not homogeneous; two cell types can be distinguished. With double and triple immunolabeling methods in combination with fluorescent latex microsphere phagocytosis assays, we have found that one population of pathognomonic cells is of glial origin and lacks phagocytic activity. We could consider this cell population specific for open NTDs. The other pathognomonic cell population is of hematogenous-monocytic origin and does have phagocytic activity. This latter group of cells forms as a result of development of NTDs. In cases of NTDs these monocytic cells occur in high number in amniotic fluid; studies are underway to clarify whether there are any processes causing appearance of pathognomonic monocytic cells in even greater numbers in amniotic fluid. We also need further confirmation of the fetal origin of the rapidly adherent cells in amniotic fluid.

The neutral red test is a simple and rapid prenatal diagnostic test for phagocytic cells in amniotic fluid; such cells are pathognomonic for open NTDs. However, the neutral red test gives no information as to the nature or origin of the phagocytic cells. The combined immunofluorescence and phagocytosis assays used here indicate that the phagocytic cells are of monocytic origin.

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