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CELL DIFFERENTIATION INCREASES ASTROCYTE PHAGOCYTIC ACTIVITY

A QUANTITATIVE ANALYSIS OF BOTH GFAP LABELING AND PAS-STAINED YEAST CELLS

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Abstract Since efficiency of phagocytic potential in activated astrocytes is still a subject of controversy, an attempt was made to quantify simultaneously phagocytic activity and astrocyte differentiation. Resorting to Junin virus, known to induce astrocyte activation, infected vs control samples of cultured rat astroglial cells were serially harvested up to day 12 post-inoculation (pi), and subjected to a triple staining procedure consisting in immunoperoxidase labeling of GFAP, periodic acid-Schiff (PAS) reaction in added baker's yeast cells and hematoxylin for nuclear staining of the whole cell monolayer. Adopting GFAP labeling as a specific marker of astrocyte differentiation, the immunoprecipitate development over time was measured. Direct calculation of the initial reaction rate was feasible given its linear behavior during the first 10 min, so that GFAP amount was regarded proportional to peroxidase activity. As determined by digital image analysis, mean optical density (MOD) values of GFAP in infected samples increased from 0.618 ± 0.082 at day 1 pi to 0.825 ± 0.125 at day 3, leveling off at 1.010 ± 0.101 as from day 9, while control uninfected samples remained unchanged at roughly 0.6 during the entire observation period. In turn, phagocytosis was quantified by PAS staining densitometry, whose intensity varied according to wall degradation of yeast cells. MOD levels of PAS-stained phagocytized yeast cells were significantly lower ($p < 0.05$) in infected vs control cultures at 48 and 72 h following their addition to the astroglial monolayer. According to simultaneous quantification of two components of astrocyte response to viral infection, it is concluded that phagocytic activity increases with astrocyte differentiation.

Resumen ***La diferenciación celular aumenta la actividad fagocítica del astrocito. Un análisis cuantitativo de GFAP inmunomarcada y de levaduras teñidas por PAS.*** Se recurrió a la cuantificación simultánea de la actividad fagocítica del astrocito en cultivo y de su grado de diferenciación celular, a fin de precisar si la activación astrocitaria influía sobre el potencial fagocítico. Primeros subcultivos de astrocitos obtenidos de encéfalo de rata, tanto controles como infectados por un virus de reconocida acción diferenciadora como el Junín, fueron seriadamente cosechados hasta el día 12 pi y sometidos a una triple tinción (inmunomarcación por peroxidasa de GFAP, reacción PAS en levaduras agregadas al cultivo, y hematoxilina para tinción de núcleos celulares). Como la velocidad de formación del inmunoprecipitado correspondiente a GFAP, marcador específico del astrocito activado, era lineal durante los primeros 10 minutos de reacción, la cantidad de GFAP detectada fue considerada proporcional a la actividad de la peroxidasa. Según determinó el análisis de imagen, los valores de densidad óptica media (MOD) de GFAP en muestras infectadas aumentaron de 0.618 ± 0.082 al día 1 pi a 0.825 ± 0.125 al día 3, estabilizándose en 1.010 ± 0.101 desde el día 1, en tanto que no se registraron cambios en los cultivos controles (MOD $\cong$ 0.6). A su vez, la fagocitosis fue evaluada por densitometría de la tinción PAS, cuya intensidad variaba en función de la degradación de la pared de las levaduras. A las 48 y 72 hs de la incorporación de levaduras a los cultivos, los valores atribuibles a células fagocitadas fueron significativamente menores en monocapas astrocitarias infectadas ($p < 0.05$). Según la cuantificación simultánea de dos componentes de la respuesta astrocitaria a la infección viral, la actividad fagocítica del astrocito se incrementa con su diferenciación celular.

Key words: astrocyte phagocytic activity, GFAP immunolabeling, image analysis, PAS staining densitometry, viral infection

Whether microglia is entirely responsible for the removal of cell detritus subsequent to CNS injury, or whether astrocytes are partly involved, is still a matter of controversy. While ultrastructural studies of induced lesions have clearly shown the phagocytic role of astrocytes[1-6], this function may only be operative to a limited degree[7, 8]. Given the availability of an in-vitro system that avoids the complexity of CNS tissues in the whole organism, astrocyte culture provides a useful tool to evaluate efficiency of its potential phagocytic activity[9-13]. In this connection, a triple staining procedure involving immunoperoxidase labeling for glial fibrillary acidic protein (GFAP), periodic acid-Schiff

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(PAS) reaction for added baker's yeast cells and hematoxylin for nuclear staining of the whole monolayer, had allowed us to determine that greater cell differentiation induced by Junin virus (JV), an Arenavirus known to accelerate astrocyte maturation both in-vivo[14, 15] and in-vitro[16, 17], is concomitant with early enhancement of phagocytic activity[12]. Therefore, measurement of GFAP expression in cultured astrocytes affords a suitable method to explore the mechanisms involved in cell differentiation and activation, as well as their relationship with astrocyte phagocytic activity.

Although GFAP immunoperoxidase labeling enables localizing the expression of such specific marker of the reactive astrocyte, its quantification in-situ requires careful monitoring of enzyme reaction. Assuming that GFAP immunoreactive mass could be estimated by correlating the appearance of the reaction product, we applied image analysis and image processing in an attempt to quantify GFAP expression in cultured astrocytes, whether infected by Junin virus or in basal conditions; concomitantly, phagocytic activity of such astrocytes was assayed by PAS staining of added baker's yeast cells as foreign bodies.

Materials and Methods

Experimental design. Astroglial cell cultures were obtained from brains of newborn Wistar rats, on the basis of previous description[3]. After 2 days of first subculture in coverslips of Leighton tubes, cell monolayers were inoculated with a mouse brain homogenate containing 10^3 (PFU) of XJ-clone 3 strain of JV, originally obtained by cloning the prototype XJ in MA cell line and then maintained by passaging in newborn mice. Matched controls were inoculated with normal mouse brain homogenate at the same dilution as the viral inoculum. Three days later, controls and infected samples whose infectivity was confirmed by plaque assay in Vero cells, were exposed to a baker's yeast cell suspension of 10^5 cells/ml during 24, 48 or 72 h and then harvested and fixed with chilled acid methanol for 10 min. The triple staining procedure mentioned above was then applied. For immunolabeling, the samples were first incubated overnight at 4 C with rabbit anti-GFAP antibody (Sigma Chemical Co, St Louis, MO), at the optimal dilution given below; the second was 1/50 dilution of biotinylated goat anti-rabbit IgG (Pel-Freez Biological, Tustin, CA) for 60 min at room temperature, and the third was 1/100 dilution of extrAvidine-Peroxidase (Sigma Chemical Co, St Louis, MO) for 30 min at room temperature. For qualitative analysis, exposure time to 0.03% diaminobenzydine tetrahydrochloride (Fluka Labs, Hauppage, NY) plus hydrogen peroxide was fixed in 30 min. Following GFAP immunolabeling, PAS reaction for added yeast cells and hematoxylin for nuclear staining of whole monolayer, were employed. Lastly, preparations were dehydrated, cleared in xylene and mounted in standard medium.

Image analysis. Quantitative analysis of both GFAP labeling of astrocytes and PAS staining of yeast cells allowed densitometric measurement of images captures by a CCD B&W Phillips video camera coupled to a Zeiss microscope at 40x magnification using a 500/510 nm filter, and digitalized by a 8-bit frame grabber at a 256-grey level. Selected images were stored in a memory file and retrieved when necessary for quantitative analysis, which was performed by means of a suitable software (Image Pro-Plus).

Statistical analysis. Mean values for each type of image processing and for each age group were investigated by Student's t test.

Electron microscopy. On the day 3 pi, astroglial cell monolayers grown in plastic flasks were scraped and centrifuged at 1800 rpm for 10 min. Pellets were fixed in 1% paraformaldehyde in Millonig buffer for 45 min, and post-fixed 90 min in a 1% solution of osmium tetroxide in the same buffer. They were then washed several times in 50% ethanol; treated with saturated uranyl acetate in 50% ethanol for 60 min; dehydrated in graded ethanols; treated with recently distilled acetone; and embedded in Vestopal. Sections were cut with glass knives, sequentially stained with uranyl acetate and lead cytrate, and examined in a Siemens Elmiskop 101 electron microscope.

Results

Optimization of GFAP labeled profile. We employed quantitative immunohistochemistry and digital image analysis to evaluate GFAP in astroglial JV-infected and control cell monolayers, since these procedures allowed us to measure GFAP immunoreactivity. To this end, monolayer areas were selected under light microscopy, thus disregarding GFAP-negative astrocytes and other cell types liable to contaminate the astroglial culture.

A blank field was captured and grey level expressed as the minimal value of optical density (OD). A dark field was then captured by turning off the light source, and grey level taken as maximum OD.

Assuming that peroxidase activity is proportional to the amount of GFAP, we monitored kinetics of immunoprecipitate formation under light microscopy. Accordingly, samples were immersed in developing solution (DAB plus H_2O_2) and a suitable dense cell area in the monolayer was selected as soon as immu-nostaining became apparent. The chosen field was then captured and digitalized at 1-min intervals, and images processed to measure the mean optical density (MOD), defined by

$$MOD = \frac{\Sigma\, OD\,(x, y)}{\text{pixel number}};$$

where OD(x, y) represents the OD of each pixel corresponding to the area under analysis.

Once a complete set of images corresponding to the total time chosen was obtained, processing was carried out as follows: the cytoplasm contour of a given astrocyte, expressly excluding its nucleus, was delimited by means of a cursor, in order to obtain an outline mask to be applied for all subsequent operations. After calculating its MOD value, the chosen cell was sequentially examined in the entire image set, and MOD plotted over 30 min.

As shown in Fig. 1, MOD increased linearly along the first 10 min, and reached a plateau towards 16 min. In

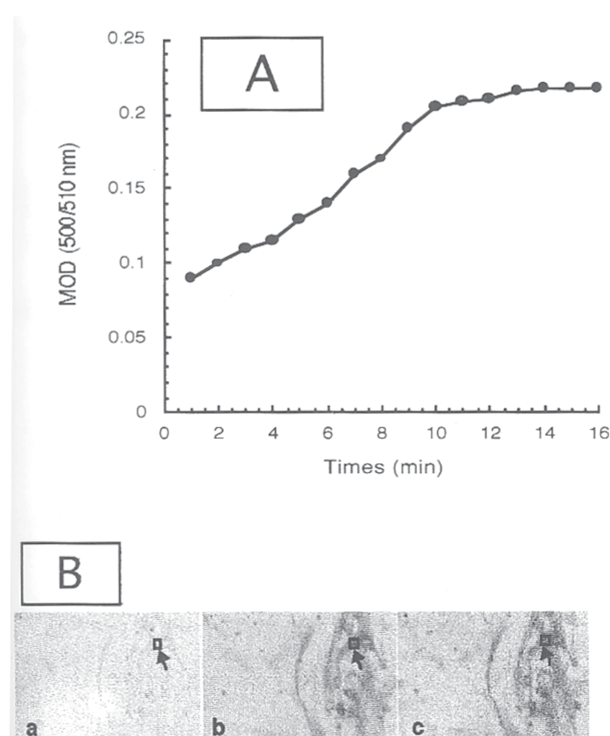


Fig. 1.– (A) GFAP immunoperoxidase labeling in a series of consecutive images captured at 1-min intervals. The first MOD measurement was taken at 1 min after the start of enzymatic reaction. (B) Computer images corresponding to the same are of astroglial culture captured at 1 (a), 5 (b) and 10 (c) min following the start of enzymatic reaction. Squares show progressive development of specific labeling in the same area, over a uniformly stained background.

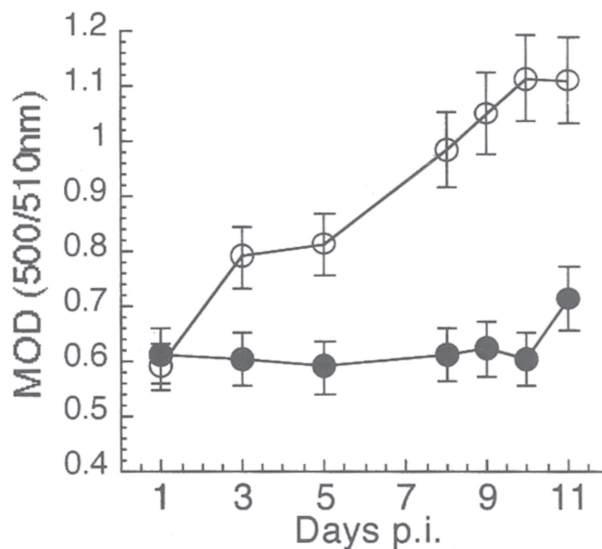


Fig. 2.– Comparison of MOD measurements of GFAP labeling at 500/510 nm in astroglial cultures infected with Junin virus (o---o) vs controls (●---●). Values are expressed as means ± SD.

the presence of excess substrate, an initial velocity rate is know to be proportional to peroxidase activity. Here, a 10-min period of development was found sufficient to maximize the peroxidase reaction rate and thus compare GFAP mass in astroglial cultures subjected to diverse experimental manipulations. Having set development conditions, monolayer areas for each experimental point were then selected for digital image processing.

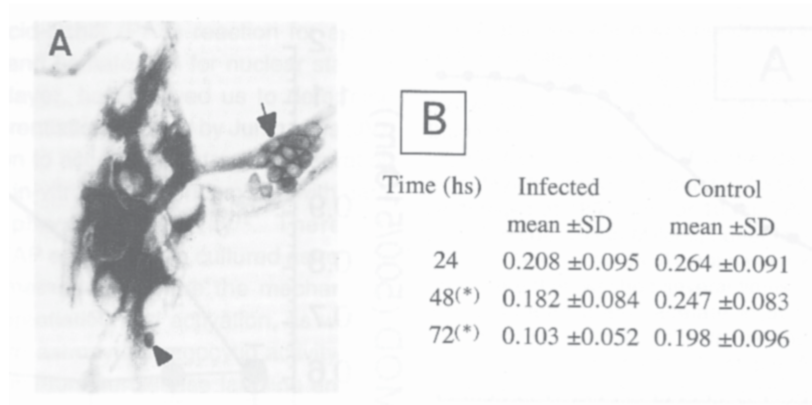
Densitometric measurement of GFAP digitalized images. After background subtraction, images were segmented by adaptive thresholding, that is by fixing the grey level range corresponding to immunolabeled areas, while software measured MOD of all pixels involved in a given area.

MOD evaluation was carried out on astroglial cultures from 1 to 11 days pi, taking the mean of 50 GFAP-positive randomly selected cells. In infected samples, MOD values increased from 0.618 ± 0.082 at day 1 to 0.825 ± 0.125 at day 3, leveling off at 1.010 ± 0.101 as from day 9, while in control cultures they remained unchanged at roughly 0.6 during the entire observation period (Fig. 2).

Densitometric measurement of PAS staining in yeast cells. As it was found that 15 min proved optimal to achieve maximal staining of yeast cells, such time was routinely used in order to rule out variations between assays.

MOD yeast measurement was performed on digitalized images of samples subjected to the triple staining procedure. Image segmentation was carried out interactively by outlining yeast cells and then measuring MOD within their perimeter. Depending on their association or lack of association to GFAP-positive astrocytes, yeast cells were regarded as intracellular or extracellular, respectively.

Yeast cells increasingly tended to associate with GFAP-positive foci, besides exhibiting changes in PAS staining intensity according to their intra or extracellular location. Fig. 3.A show that yeast cells localized within an astrocyte displayed weak PAS staining, whereas extracellular yeast cells presented stronger and more uniforme PAS positivity. Thus, the lysis of yeast walls by lysosomal enzymes would presumably be reflected by the degree of PAS staining. In order to evaluate this differential staining pattern, we resorted to digital image analysis. MOD values of yeast cells at 1, 2 and 3 days following their addition to control and JV-infected cultures were compared. Although mean MOD values decreased in both (Fig. 3.B), Student's t test disclosed that PAS staining was significantly weaker ($p < 0.05$) in infected cultures vs controls at 48 and 72 h after incubation with yeast cells.



Time (hs)	Infected mean ±SD	Control mean ±SD
24	0.208 ±0.095	0.264 ±0.091
48(*)	0.182 ±0.084	0.247 ±0.083
72(*)	0.103 ±0.052	0.198 ±0.096

Fig. 3.– (A) Photomicrograph of astroglial cell monolayer after 72 h incubation with yeast cells. Intracellular yeast cells (arrow) display weaker PAS staining than those outside astrocytes (arrowhead), which are more strongly and more uniformly stained. x 1 100. (B) Comparison of MOD values in yeast cells after incubation (24, 48 and 72 h) with Junin virus-infected and control cultured astrocytes. Values correspond to the mean of 100 intracellular yeast cells.
* Significantly different vs controls ($p < 0.05$).

Besides, electron microscopy (not shown) disclosed differences in cell wall thickness of yeast cells subjected to astrocyte phagocytic activity, whether intact in extracellular or disrupted in intracellular location.

Discussion

According to results obtained, simultaneous quantitative evaluation was achieved of two components involved in astrocytic response to CNS injury: on one hand, cell differentiation as depicted by GFAP expression, and on the other, the characterization of phagocytic activity as disclosed by the intensity of PAS staining of added yeast cells.

Resorting to digital image analysis, peroxidase activity was evaluated along GFAP labeling of cultured astrocytes. The fact that our proposed quantitative system provides the 256-grey value required for minimal accuracy in cytophotometry, allowed us to detect differences which could not be discerned in a previous report [12], where no attempt was made to determine peroxidase kinetics in GFAP. On the basis of the approach applied by Jonker et al.[18] in their analysis of regional differences in the activity of glutamate dehydrogenase in rat liver sections by means of monitoring the colored final reaction product in time, for the present case we adopted as a working hypothesis that peroxidase activity is proportional to the amount of GFAP. When monitoring the kinetics of the reaction under light microscopy, we found that immunoprecipitate formation increased linearly during the first 10 min, enabling direct calculation of the initial reaction rate in JV infected vs control samples.

Since astrocyte phagocytic activity was assayed by the addition of baker's yeast cells whose PAS staining varied according to cell wall degradation, MOD measurement of PAS intensity was the complementary parameter employed. The finding that MOD values were influenced by viral infection and yeast cell incubation, would seem to rule out the presence of any staining artifact.

To conclude, the present work illustrates how a conventional immunocytochemical method plus a traditional staining procedure as PAS, may become a highly accurate tool when performed jointly with digital image analysis, thus validating that phagocytic activity increases with astrocyte differentiation. Although triggering of astrocyte activation was experimentally induced by a virus known by its differentiating effects on such cell type, a phagocytic activity concurrent with microglial role in clearance of damaged tissues, deserves to be investigated in other viral o non-viral CNS infections. Furthermore, given the outcome of astrocytosis involving an enhancement of its own phagocytic activity, it is of absorbing interest to characterize the dynamics of this phenomenon in all forms of CNS injury. In this connection, findings achieved by Cheng et al.[19] suggested that, in response to decortication, reactive astrocytes are the primary cells responsible for removing degenerating axon terminals, and that the coordinate increase in GFAP may serve to stabilize the extension of reactive astrocytic processes during phagocytosis. Moreover, as hypothe-sized in a thorough review on neuron-glial and glial-glial interactions[20], the control of induction and progression of glial response seems critical for the outcome of neurotrauma, brain ischemia and chronic neurodegenerative diseases among others, since specific functional properties of activated cells are deter-

minants of neuron survival, axon regeneration and synaptic plasticity.

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Creativity in science, as in the arts, cannot be organised. It arises spontaneously from individual talent. Well-run laboratories can foster it, but hierarchical organisation, inflexible, bureaucratic rules, and mountains of futile paperwork can kill it. Discoveries cannot be planned; they pop up, like Puck, in unexpected corners.

La creativida en ciencia, como en las artes, no puede organizarse. Surge espontáneamente del talento individual. Laboratorios bien organizados pueden fomentarla, pero organizaciones jerárquicas, inflexibles, con reglas burocráticas, y montañas de papeles administrativos pueden aniquilarla. No se pueden planear los descubrimientos; aparecen, como Puck, en rincones imprevistos.

Max Perutz

I wish I'd made you angry earlier. Essays on Science and Scientists. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1998, p IX