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Reduction of the microglial cell number in rat primary glial cell cultures by exogenous addition of dibutyryl cyclic adenosine monophosphate

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Abstract

The present work examined the effects induced by dibutyryl cyclic adenosine monophosphate (dB–cAMP) on microglial cells in primary glial cell cultures from newborn rats. Microglial cells were identified by OX42 immunohistochemistry and nucleoside diphosphatase histochemistry. Double staining for astrocytes was carried out by combination with glial fibrillary acidic protein immunolabeling. Addition of 0.25 mM dB–cAMP to the cultures decreased the microglial cell number about sixfold. The findings suggest that the effect of dB–cAMP on the microglial cells might be either a direct action of dB–cAMP on the microglial cells or an indirect effect mediated by the astroglial cells.

Keywords: Cell cultures; dB–cAMP; Microglia; Nucleoside diphosphatase; OX42; Glial fibrillary acidic protein

1. Introduction

In the normal central nervous system (CNS) astrocytes serve important functions in the metabolism and neurotransmission of neurons (Hertz, 1991) while microglial cells are generally considered to serve as dormant immune surveillance cells, which however, are potentially the most important element for development of CNS immune responses (Graeber and Streit, 1990; Thomas, 1992). This inherent difference in their normal functions is also reflected in the different expression of immunoregulatory molecules and cytokines of both cell types following brain lesioning (Eddleston and Mucke, 1993; Finsen et al., 1993a,b; Morgan et al., 1993; Lehrmann et al., 1995) as well as in their cytokine response to in vitro stimulation with for example lipopolysaccharide (LPS) (Sawada et al., 1989; Yao et al., 1992; Lee et al., 1993a,b), interleukin-1 beta (IL-1β) (Lee et al., 1993b), and interferon-gamma (IFN-γ) (Sawada et al., 1993). The fact that these cells are subject to different regulatory mechanisms in vitro is documented by the findings that IFN-γ induced MHC class II antigen expression (Wong et al., 1985; Frei et al., 1987; Suzumura et al., 1987; Sasaki et al., 1989; De Groot et al., 1991) is suppressed in astrocytes but not in microglial cells by IL-1β (Smith et al., 1993), glutamate (Lee et al., 1992), as well as by cyclic adenosine monophosphate (cAMP) (Sasaki et al., 1990) and norepinephrine, which also increases intracellular cAMP concentrations (Frohman et al., 1988).

In accordance with a previous report (Castellano et al., 1991), primary mixed glial cell cultures contain both the ramified and the ameboid microglial cell type (RM and AM, respectively), the RM closely adhering to the astrocyte monolayer, and the AM laying more superficially and less attached to the astrocytes. In addition, we found (Dalmau et al., 1992b) that the distribution of RM and AM was not homogenous but related to astroglial cell density, suggesting that astroglial cells in the culture dishes may determine the differentiation of microglia.

Based on the above observations and the knowledge that dibutyryl–cAMP (dB–cAMP) has some biological effects on cultured astroglial cells (Juurlink and Hertz, 1985; Le Prince et al., 1991; Bruce et al., 1992), we determined to investigate (1) to which extent dB–cAMP would influence the morphology or the numerical density of microglial cells in primary mixed glial cultures, and (2)

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whether such differences might correlate with variances in the regional density of astroglial cells.

2. Material and methods

2.1. Preparation of cell cultures and fixation

Fourteen-well primary glial cell culture plates were prepared from neopallium of newborn Wistar rats in accordance with a modification of a previously described method (Hertz et al., 1985). This procedure was repeated two times. The neocortex of one day old postnatal rats was aseptically dissected out and the meninges and blood vessels were carefully removed. Following criss-cross cutting in a medium containing 20% horse serum, the suspension was filtered through a 80 μm filter and transferred as a single cell suspension to 35 mm culture dishes (Nunc) with coverslips coated with poly-D-lysine, using 1.5 ml medium per dish. The cultures were incubated at 36.5°C in an atmosphere containing 5% CO_2. The culture medium consisted of Minimum Essential Medium Eagle (Gibco, Cat. No. 21430-020) supplemented with L-glutamine, amino acids, vitamins, penicillin, gentamicin, sodium bicarbonate and 20% heat-inactivated horse serum. The medium was changed twice a week. After 1 week, the concentration of horse serum was reduced to 10%. After sixteen days and for each time that the procedure was repeated, half of the cell cultures were treated with 0.25 mM dB–cAMP in the medium, while the rest of the cultures (dB–cAMP untreated) were used as controls. Ten to twelve days later, treated and untreated cultures were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) with 5% sucrose for 2 h at 4°C. Afterwards, the cultures were rinsed in cacodylate rinse buffer (CRB) (0.1 M cacodylate buffer at pH 7.4 with 7.5% sucrose) (2×10 min).

2.2. Specific microglial staining

Microglial cells were visualized using avidin-biotin immunohistochemistry, using the OX42 primary mouse antibody against the rat complement receptor type 3 (CR3) (Seralab; UK) or by histochemical staining for nucleoside diphoshatase (NDPase) demonstration. Simultaneous double labeling of microglia and astroglia was achieved by double staining, as reported by Castellano et al. (1991).

OX42 immunohistochemistry was carried out as follows. Preceding an initial wash in 0.05 M Tris-buffered saline at pH 7.4 (TBS) (2×10 min) and then in TBS with 1% Triton X-100 (3×15 min), the cell cultures were incubated in 10% fetal calf serum (FCS) diluted in TBS for 30 min at room temperature (RT). The incubation with the primary mouse anti-CR3 antibody (Seralab; UK) was performed overnight at 4°C in a dilution of 1:600 in TBS with 10% FCS. Following a rinse in TBS with 1% Triton X-100 (3×10 min), the cell cultures were incubated at RT for 60 min with an anti-mouse Ig-biotinylated antibody (Amersham; UK) diluted 1:200 in TBS with 10% FCS. After washing in TBS with 1% Triton X-100 (3×10 min) the samples were incubated for 60 min at RT in a 1:70 dilution of avidin labeled peroxidase (Sigma) in TBS with 10% FCS and then in TBS with 1% Triton X-100 (2×15 min) and in TBS (2×15 min). To visualize the peroxidase, 3,3′-diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen (50 mg DAB in 100 ml 0.05 M TBS, pH 7.4, with 33.3 μl H_2O_2 added just before use). After a final rinsing in TBS ($2 \times$ for 10 min) the cell cultures were coverslipped with Glycergel® (Dako; DK).

Demonstration of NDPase enzymatic activity was performed as reported by Castellano et al. (1991). Briefly, the culture dishes were incubated at 38°C for 25 min in a medium containing 7 ml of distilled water, 80 mM Trizma maleate buffer (Sigma; T-3128, pH 7.4), 5.05 mM $MnCl_2$, 3.62 mM $Pb(NO_3)_2$, and 2.16 mM sodium salt of inosine 5′-diphosphate (Sigma; Cat. No. I 4375) as substrate. After incubation, the culture dishes were rinsed (3×10 min) in CRB, treated in 2% $(NH_4)_2S$ for 2 min, rinsed in CRB (2×10 min) and distilled water (2×1 min) and then immersed in 1% $AgNO_3$, rinsed again in distilled water (2×1 min) and finally coverslipped with Glycergel® (Dako; DK).

2.3. Double labeling technique

Simultaneous demonstration of microglial and astroglial cells was achieved through three sequential steps. The histochemical reaction for microglial NDPase was first performed as described above but without the final ammonium sulphide treatment. GFAP immunohistochemistry was then performed as described for OX42 immunohistochemistry (Castellano et al., 1991), using a primary rabbit anti-GFAP antibody (Dako; DK) in a concentration of 1:2400, and an anti-rabbit Ig-biotinylated second antibody (Amersham; UK) diluted 1:300. The last steps were the visualization of the histochemical and immunohistochemical reaction products. Firstly, the immunohistochemical reaction was performed by immersion of the cell cultures in 1-naphtol solution with 0.01% H_2O_2 for 15 min at RT. The naphtol solution was freshly prepared by dissolving 50 mg of 1-naphtol (Sigma) in 0.5 ml ethanol and adding this, together with 10 ml 1% $(NH_4)_3CO_3$, to 89.5 ml of TBS. The solution was filtered before addition of the H_2O_2. After rinsing in CRB (2×10 min), the NDPase histochemistry was visualized by treatment in 2% $(NH_4)_2S$ for 2 minutes. Afterwards, the cell cultures were again rinsed in CRB (2×10 min). Finally, cell cultures were treated in 0.05% Azur A in 0.05 M TBS (pH 8.0) for 15 min. After being washed in distilled water (2×2 min), the cell cultures were immersed in 1% $AgNO_3$, washed again in distilled water (2×2 min) and finally coverslipped with Glycergel® (Dako; DK).

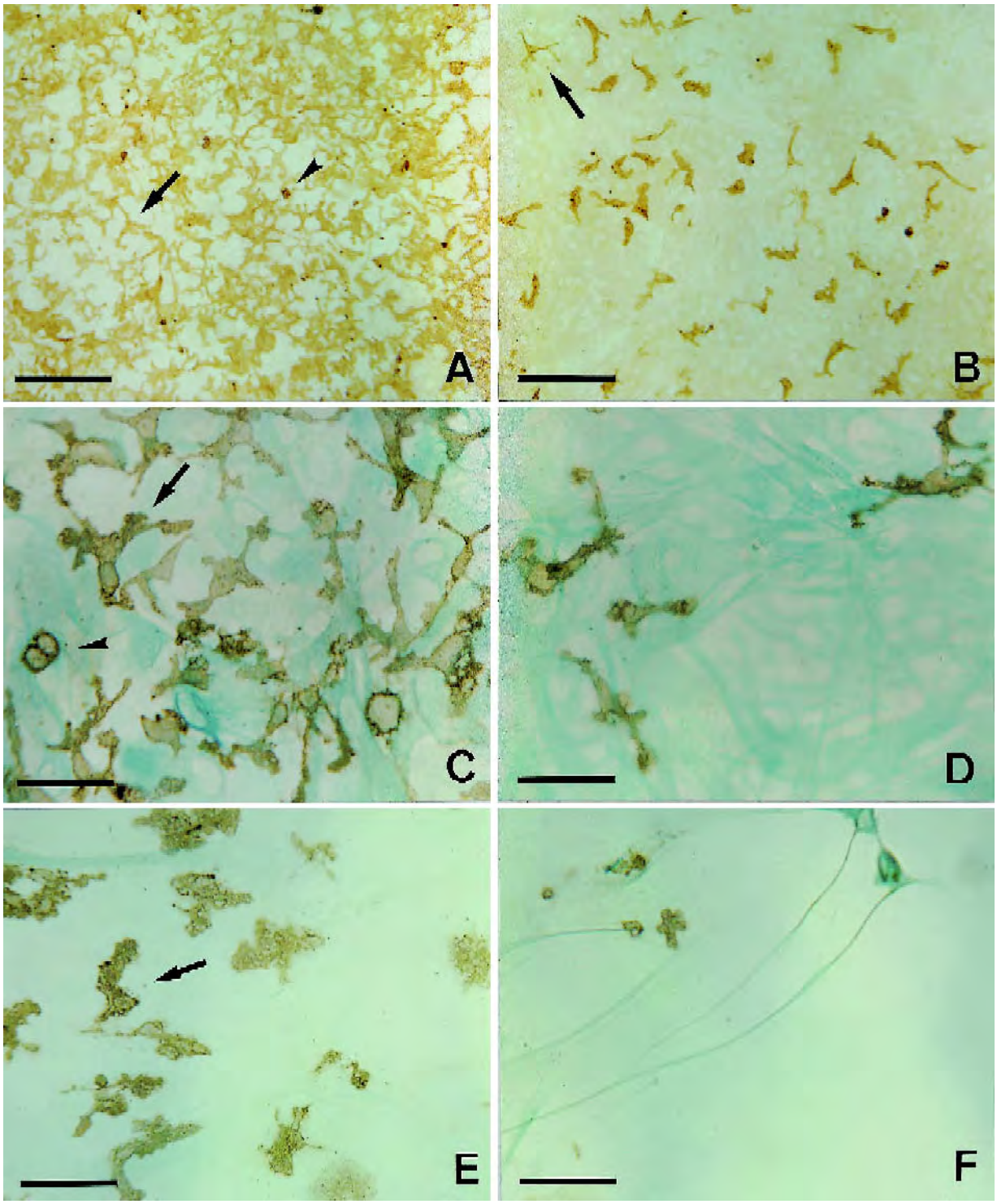

Fig. 1. Primary glial cell cultures stained with either OX42 immunohistochemistry (A, B) or NDPase/GFAP double labeling (C–F) corresponding to untreated cell cultures (A, C, E) and dB–cAMP treated cell cultures (B, D, F). Untreated cell cultures (A) showed a significant number of ramified microglia (arrow) but less of ameboid microglia (arrowhead) homogeneously distributed. Treatment with dB–cAMP (B) produces a reduction of microglial cell density, in particular affecting ramified microglial cells (arrow). Double labeling for NDPase (brown) and GFAP (blue) in untreated cell cultures (C) demonstrates that ramified microglia (arrow) are in close relationship with astrocytes. Pairs of round microglia (arrowhead) suggesting cell proliferation were also found. DB–cAMP treated cell cultures (D) showed not only a reduction of microglial cells but also a strong increase in GFAP immunoreactivity and, thinner and longer astroglial cell processes. In astrocyte-free areas of untreated cell cultures (E), NDPase positive ramified microglia-like (arrow) and ameboid microglia were observed and the addition of dB–cAMP in the cell cultures (F) also reduces the microglial cell density affecting both microglial cell types. A, B: bar = 125 μm; C–F: bar = 50 μm.

2.4. Cell count

Statistic analysis was carried out from six randomly chosen dB–cAMP untreated dishes and six randomly chosen dB–cAMP treated dishes. Microglial density values were obtained by counting enzyme histochemical stained bodies using a ×40 objective from 10 fields in the astrocyte-rich area and 10 fields in the astrocyte-free area for each cell culture. Each field was limited within a square of 250 μm by 250 μm. ANOVA test was performed at the 95% significance level in order to contrast differences.

3. Results

As previously reported by Castellano et al. (1991), stainings by OX42 immunohistochemistry and NDPase enzymehistochemistry revealed the presence of a significant number of microglial cells in the primary glial cell cultures. No discernible differences were noticed between cultures stained with OX42 immunohistochemistry or NDPase enzymehistochemistry, indicating that both techniques stained the same population of cells. The dB–cAMP treatment did, however, result in changes with regard to microglial cells.

3.1. Untreated cultures (control)

Microglial cells in untreated cell cultures usually appeared as ramified cells with moderate levels of OX42 and NDPase labeling, homogeneously distributed in the cultures (Fig. 1A). These ramified microglial (RM) cells, resembling the ramified type of microglia in vivo, displayed a pleomorphic cell body with a variable number of branching processes, sometimes with a spiny-like appearance. The cells were usually found as isolated cells without forming cell clusters. Apart from the RM, a number of ameboid microglia (AM) were also distinguished (Fig. 1A). Ameboid microglial cells were smaller than RM and displayed pseudopodia and/or filopodia instead of cell processes. In addition, the AM cells showed a higher level of both OX42 and NDPase staining. Finally, round shaped NDPase/OX42 labeled cells, and pairs of these cells suggesting active cell proliferation, were frequently found (Fig. 1C). These cells displayed a slightly higher OX42/NDPase staining compared to the other OX42/NDPase positive cells. Double labeling, allowing simultaneous visualization of microglia and astroglia, revealed that RM were distributed among the polygonal GFAP stained astrocytes (Fig. 1C). The microglial cell bodies and processes thus intermingled with astrocytes in the same layer. In contrast to this, AM were always found on the top of the confluent layer of astrocytes and RM. Finally, we should note that microglial cells were also present in areas without astrocytes although with a slightly lower cell-density (Fig. 1E). In these astrocyte-free areas two populations of cells were found: intensely stained AM cells, a few of which bore scarce thin branches, and a group of pleomorphic cells which we termed as ramified microglia-like (RM-like) cells. Ramified microglial-like cells showed a moderate OX42 immunoreactivity and NDPase activity and were mostly recognized as flattened pleomorphic cells resembling the RM cell type, but some others showing a roundish shape or large round shape with a flat halo around the soma. These cells appeared to be attached directly to the culture dishes or coverslips and they did not display any special distribution.

3.2. DB–cAMP treated cultures

In dB–cAMP treated cultures a decrease in microglial cell density was easily noticed (Fig. 1B, D). Microglial cell density in dB–cAMP treated cultures was about 83% lower than in non-treated cell cultures, mostly affecting the ramified cell population (Table 1). After dB–cAMP treatment, RM and AM mean reduction in the astrocyte-rich area was about 84% and 77% respectively, and about 89% and 39% in the astrocyte-free areas (Table 1). Remaining RM showed unchanged moderate OX42/NDPase staining and unchanged morphology. Pairs of cells suggesting cell division, as previously described, were uncommon. Ameboid microglia (AM) retained, as in the untreated cultures, a random distribution and strong OX42/NDPase staining.

Table 1
Estimation of microglial cell density (number of cells/field) in the untreated and dB–cAMP treated primary glial cell cultures

	Total of microglia [a]	Astrocyte-rich area			Astrocyte-free area		
		RM [b]	AM [c]	RM + AM	RM-like [d]	AM [c]	RM-like + AM
Untreated	59.9 ± 11.2	138.6 ± 10	17.5 ± 6.9	78 ± 19.1	72 ± 9.1	11.5 ± 3.5	41.7 ± 10.2
Treated	10.5 ± 2.3 *	23 ± 6.7 *	4.1 ± 0.4 *	13.5 ± 4.2 *	8 ± 3.3 *	7 ± 1.7	7.5 ± 1.8 *
ERP [e]	83%	84%	77%	83%	89%	39%	82%

* $P < 0.05$ as compared to values in control primary glial cell cultures (analysis of variance and Scheffé test).
[a] Total number of microglia consisted of ramified microglia and ameboid microglia in astrocyte-rich areas as well as ramified microglia-like and ameboid microglia in astrocyte-free areas.
[b] Ramified microglia.
[c] Ameboid microglia.
[d] Ramified microglia-like.
[e] Estimated reduction percentage.

Double staining for GFAP showed that astroglial cells, as expected after dB–cAMP treatment, displayed a strong increase in GFAP immunoreactivity, in addition to morphological changes characterized by thinner and longer cell processes. DB–cAMP-treatment did not alter the inter-cellular organization within the cultures, as the RM remained located in the same layer as the differentiated astroglial cells, while AM were found on the top of the layer. In the astroglial free-areas intensely stained AM cells (Fig. 1F) and RM-like cells were reduced, affecting mostly the latter.

4. Discussion

In the present study we demonstrated that microglial cells responded to the addition of dB–cAMP in primary rat mixed cell cultures. The mean RM cell density in the astrocyte-rich area decreased about sixfold in the dB–cAMP treated cell cultures, while AM in the same area were reduced fourfold. In the astrocyte-free area RM-like and AM cell density were decreased about ninefold and less than twofold, respectively.

Although dBcAMP effects on microglia have been reported by some authors (Bowman et al., 1992; Righi, 1993), no previous studies using selective cell markers have shown changes in microglial cell density in response to dB–cAMP treatment in vitro. It has been reported, however, that a reduction in cell number was observed in cultures of macrophages derived from peripheral blood monocytes when dB–cAMP were provided (Peters et al., 1990). In addition, exogenous addition of dB–cAMP was found to induce transformation of mature macrophages into dendritic cells with loss of attachment ability, which is a phenotype devoid of most typical macrophage characteristics (Peters et al., 1990). As already known, ramified or resting microglia are regarded as an intrinsic and mature macrophage population of the CNS (Del Río Hortega, 1932; Perry and Gordon, 1991; Ling and Wong, 1993). It is generally accepted that dB–cAMP exerts its effects by raising the intracellular levels of cAMP, acting as a second messenger with a crucial role for cell activation and differentiation. Therefore microglial cells may be affected by changes in the intracellular levels of cAMP in terms of differentiation. Thus change in cell adherence properties could explain the reduction in RM cell number, although a reduced proliferation rate and an increased cell death should be considered. In vivo studies have already suggested that apoptotic cell death is a mechanism for population control of microglia (Gehrmann and Banati, 1995). Moreover, the induction of apoptosis by intracellular cAMP elevation has been demonstrated in resting cells (Lømo et al., 1995), suggesting that cAMP, as second messenger, plays an important role in apoptosis (see Gold et al., 1994; Vermes and Haanen, 1994). Cyclic AMP-induced apoptosis is a calcium-dependent process and involves the activation of a cAMP-dependent protein kinase I (Gruol et al., 1989; McConkey et al., 1990; Lanotte et al., 1991). Although there is some disagreement about the exact chain of events in this process, specific changes in gene expression have been shown to be responsible for apoptosis (see review Vermes and Haanen, 1994).

In spite of a direct action of dB–cAMP on the microglial population, the reduction in cell number could be mediated indirectly through the astroglial cell population. Although there are some discrepancies on the effect of dB–cAMP on the morphological differentiation in cultured astroglial cells (MacVicar, 1987), it is generally accepted that dB–cAMP induces biochemical changes and inhibition of growth rate on these glial cell type in vitro (Juurlink and Hertz, 1985; Le Prince et al., 1991; Bruce et al., 1992). Moreover, microglial proliferation and differentiation in vitro is mediated by astrocyte derived factors (Frei et al., 1986; Suzumura et al., 1991; Ganter et al., 1992). Also, in vivo, the differentiation of RM from primitive ramified microglial precursors occurs in close relationship to astroglial differentiation (Dalmau et al., 1992a,b), supporting the view that astrocytes regulate the microglial phenotype by the release of certain compounds or by mechanism of cell to cell contact (Benveniste, 1993; Piani et al., 1994). The double labeling staining of the cultures with NDPase and GFAP enabled us to observe that typical RM were found only in relation to astroglia, whereas in the areas where astroglia were absent microglial cells always displayed roundish shapes with a few processes. This would suggest that the expression of RM cell phenotype and even microglial properties such as proliferative capacity could be highly dependent on either close physical presence, soluble factors or the differentiational stage of the adjacent astroglia.

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