REVIEW

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The role of flow cytometry in the study of cell growth in the rat anterior pituitary gland

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SUMMARY

Flow cytometry is a suitable technique for studying *in vivo* and *in vitro* the cell cycle kinetics of different animal and human tissues, both in normal and tumoral conditions. The rat anterior pituitary gland is a model to investigate cell growth and replication of differentiated, neuroendocrine cells, and we report current evidence on its cell cycle kinetics as well as on the role played by flow cytometry in this type of study. The proliferation potential of normal anterior pituitary cells is related to a number of different conditions, including heterogeneity of cell types, age and sex of donors, and circadian influences. In addition, the trend of cell proliferation in both *in vivo* and *in vitro* studies is similar, suggesting that cultured anterior pituitary elements may, at least in parts, retain growth features analogous to those of the intact gland. Sorting of selective cell types and analysis of the relation between proliferating anterior pituitary cells and the light-dark cycle have shown that flow cytometry may be useful to investigate the replication process of the gland. By using a combination of flow cytometry, light microscopic immunocytochemistry and morphometry, we have reported a peculiar trend of proliferation in primary monolayer cultures of rat anterior pituitary gland, characterized by a non-linear reduction in their proliferation rate with advancing age, primarily dependent on a reduced transition of cells from the G0/G1- to the early S-phase pool. These studies indicate that flow cytometry offers insights into cell cycle check points of anterior pituitary cells, and suggest that it might be applied to the study of growth of selective pituitary elements, both in normal and tumoral conditions.

INTRODUCTION

Flow cytometry is a powerful analytical tool that allows a multiparametric study of cells in flow, where the combination of fluorescence and scattering signals gives the possibility of a very accurate quantitative and, to a more limited extent, qualitative analysis of cellular characteristics and functions (Shapiro 1988; Jaroszeski and Radcliff, 1999; Cunnigham, 1999). Flow cytometric analysis of the cell cycle is usually performed by measuring either the DNA, DNA-RNA and DNA-protein content or DNA and cycle-related antigens of cultured cells. The monoparametric measurements of DNA content on fixed, RNAse treated cells,

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stained by DNA specific dyes are often hampered by the frequent lack of a clear-cut distinction between the different cell-cycle phases, that always overlap to a certain extent. This problem has been overcome by the biparametric DNA/ bromodeoxyuridine (BrdU) analysis. Monoclonal antibodies (mAb) to the synthetic thymidine analogue, BrdU are used for its detection by direct or indirect immunofluorescence techniques in optical microscopy or flow cytometry (Gratzner 1982). As BrdU labels DNA replication sites in the interphase cell nucleus (Nakayasu and Berezney 1989), the anti-BrdU mAb specifically stains cells actively synthesizing DNA. BrdU has now been largely used in both *in vivo* and *in vitro* systems, and we have sucessfully applied a direct immunofluorescence technique in flow cytometry to the quantitation of cells incorporating BrdU as Friend cells (Papa *et al.* 1988), K562 tumor cell line (Vitale *et al.* 1989b), murine erytholeukemia cells (Mazzotti *et al.* 1990), murine melanoma cells treated with DNA synthesis inhibitors (Vitale *et al.* 1990), hepatocytes of regenerating rat liver (Vitale *et al.* 1991) and Daudi cells (Di Pietro *et al.* 1997). Using a fluorescent, intercalating dye, usually propidium iodide (PI), a counterstaining of the double stranded DNA is achieved, allowing simultaneous detection of both diploid and tetraploid cells as well as BrdU-labeled cells throughout the different phases of the cell cycle.

In addition to a biparametric fluorescence (PI and BrdU) analysis, flow cytometry may exploit signals deriving from scatter of the incident laser light, as the Forward (FSC) and Side (SCC) Scatter (Shapiro 1988). These two parameters are basically dependent on cell morphology, and we demonstrated that they can be related to cell cycle progression (Vitale *et al.* 1989b), chromatin conformational transitions (Papa *et al.* 1987, Santi *et al.* 1987, Cocco *et al.* 1988) and density of intracellular interfaces (Vitale *et al.* 1989a).

In this review, we report current data on cell growth in the rat anterior pituitary gland which is one of the most widely used animal models for the study of cell function in neuroendocrine tissues, and focus on available evidence concerning the application of flow cytometry to the evaluation of cell cycle kinetics in adenohypophysial cell types. Knowledge of the different phases regulating the replication process of the normal pituitary cells seems very important to understand which steps of the cell cycle might be reponsible for initiation of growth in differentiated cells, which might trigger expansion of selective cell types or prevent their unrestrained proliferation. In addition, since the anterior pituitary is easily subjected to benign neoplastic transformation of selective cell types (Reichlin 1991) leading in humans to adenoma formation and important clinical syndromes (Toni *et al.* 1997), cell cycle analysis of specific pituitary cell populations might reveal selective molecular targets for therapeutic agents able to inhibit the survival of adenomatous cell clones, providing new tools to cure the associated endocrine syndromes in humans.

PROLIFERATIVE FEATURES OF THE RAT ANTERIOR PITUITARY CELLS

In vivo studies

The rat anterior pituitary gland shows a progressive decrease in the rate of cell proliferation from birth to maturity, with a peak estimated between 1-2% of the total cellular population around 30 days of postnatal life, depending on the technique of investigation used, and a plateau of 0.7-0.8% proliferating cells at 60-70 days of age (Valotaire and Duval 1972, Shirasawa and Yoshimura 1982, Carbajo-Perez and Watanabe 1990, McNicol and Carbajo-Perez 1999). As a result of this process, the total number of pituitary cells increases by 25-fold from day 10 to day 120 after birth; in contrast, within the same period of time the gland weight rises 42 times (Friend 1979). Since the average cellular size increases by 30% during the first 30 days of life (Carbajo-Perez and Watanabe 1990), factors other than rate of proliferation appear determinant to the growth of the entire gland, including cell hypertrophy (Carbajo-Perez and Watanabe 1990) and development of the vascular network (Carbajo *et al.* 1991). In addition, sex plays an important role: cell proliferation is lower in males than in females (Crane and Loomes 1967, Oishi *et al.* 1993), and fluctuates throughout the female reproductive cycle, peaking in estrus (Hunt 1942, Hunt 1943).

Circadian variations in the proliferation activity have also been reported, appearing dependent on the technique used, and gender. Initial studies in

male rats showed prominent peaks of mitosis in the morning (Nouet and Kujas 1975), consistent with recent data showing a maximum mitotic rate around noon and a minimum around midnight (McNicol and Carbajo-Perez 1999). In contrast, other studies pointed to an increased proliferation activity at night (Oishi *et al.* 1993). In female rats, a mitotic peak has been identified in the early morning (Hunt 1943). These data suggest that, although the light period contributes to trigger a wave of cells that replicate their DNA, photic stimuli are not primary determinants in cell replication of the rat anterior pituitary. Other factors, such us hypothalamic and peripheral hormonal influences, as well as paracrine and/or autocrine mechanisms, might drive synchronization in the replicating cellular populations. In any event, a single replication wave seems to occur within a time frame of around 24 hours, as expected from the evidence that the zenith and nadir of mitoses stay 12 hours apart (McNicol and Carbajo-Perez 1999). Since the S-phase of the pituitary cell cycle has been calculated to be about 11 hours, and the G2/M phase occupies roughly 3 hours, the most variable and cell type-dependent part of the replication process has been presumed to be G1 (McNicol and Carbajo-Perez 1999), consistent with the differential distribution of cell cycle enzymes in different pituitary cell types (Qian *et al.* 1997, Qian *et al.* 1998).

In mammals, the cellular population of the anterior pituitary shows a differentiated state early during the embryonic period (Voss and Rosenfeld 1992, Asa and Ezzat 1999), resulting in adult glandular parenchyma rich in unique epithelial, mesenchymal and endothelial elements (Lechan and Toni 2000). These cells produce a variety of substances acting through paracrine and/or autocrine modalities (O'Halloran *et al.* 1990, Houben *et al.* 1990, Jones *et al.* 1990), part of which have been shown to selectively regulate growth and differentiation of specific cell types (Pawlikowsky 1999). In rodents, in fact, cellular proliferation primarily involves division of mature, differentiated cells (Mastro *et al.* 1969b, Kurosumi 1971); however, recruitment of undifferentiated precursors might also be possible, but no proof for a pituitary stem cell is yet available.

Epithelial cell types

Fluctuations in the relative proportions of different epithelial cell types have been reported to occur from birth to adult life: mature and immature acidophils, i.e. growth hormone-containing cells (somatotrophs), prolactin-containing cells (lactotrophs) and possibly, common precursor cells (mammosomatotrophs), show a progressive increase in their replicating rate, reaching the highest mitotic activity at day 70 after birth, when they account for around 80% of dividing cells (Shirasawa and Yoshimura 1982) Oishi *et al.* 1993). However, whereas some studies report that proliferating somatotrophs are at least four times more numerous than lactotrophs (Shirasawa and Yoshimura 1982), other studies indicate that lactotrophs prevail (Oishi *et al.* 1993). In contrast, around 20% of newborn, total mitotic cells are basophils, half of which are thyroid-stimulating hormone-containing cells (thyrotrophs) and the other half are follicle-stimulating hormone- and luteinizing hormone-containing cells (gonadotrophs). Interestingly, replicating thyrotrophs (Fig. 1A-1B) progressively reduce their percentage to undetectable levels (Siperstein *et al.* 1956, Shirasawa and Yoshimura 1982), as opposed to proliferating gonadotrophs whose number remains, although low, relatively constant throughout the first two months of life. A distribution similar to thyrotrophs is observed for adrenocorticotropin-releasing hormone-containing cells (Shirasawa and Yoshimura 1982). Also, immunonegative cells undergo a continuous decline in their replication rate with advancing age, but their trend of proliferation is always very substantial, accounting for one third at early postnatal times to around 10% at day 70 of life, respective to the total dividing cells (Shirasawa and Yoshimura 1982). Differences in proliferation percentages among the various cell types with increasing age have been also reported using *in vivo* administration of BrdU (Carbajo-Perez and Watanabe 1990, Carbajo *et al.* 1991, Oishi *et al.* 1993) and, despite some inconsistencies with the previous mentioned studies, confirms that the proliferation potential of the epithelial cells is strictly age-dependent.

Sex differences may primarily involve specific cell types: in male rats, proliferation of lactotrophs reaches a peak at day 20 after birth, whereas in females a similar peak is reached at day 60 of postnatal life (Takahashi *et al.* 1984). It is possible that these differences reflect induction of apoptosis following decline in circulating estrogen con-

centrations in male- (Drewett *et al.* 1993) with respect to female rats. In addition, it is apparent that in females, all different replicating cell types increase their proliferation activity at estrus with respect to other phases of the reproductive cycle (Oishi *et al.* 1993).

Mesenchymal and endothelial cell types

Very few data are available on these cell types. Indirect evidence, based on agranularity and morphological features, suggests that the folliculostellate cells reduce their proliferation activity from neonatal to adult life, shifting from around 25% to less than 10% of total mitotic cells, respectively (Shirasawa and Yoshimura 1982). However, some evidence has suggested that a larger number of them might be present at maturity (Oishi *et al.* 1993). In contrast, more direct data using specific immunocytochemical markers indicate that their replication rate would be, as a whole, substantially lower than that previously suggested (Carbajo-Perez and Watanabe 1990). Indirect data also indicate that endothelial cells of the rat pituitary vessels proliferate only during the first two weeks of postnatal life (Carbajo *et al.* 1991), although some replication of endothelial elements has been suggested to occur at maturity (Oishi *et al.* 1993).

***In vitro* studies**

As the rat anterior pituitary cells retain their differentiated state *in vitro*, primary monolayer cultures of rat anterior pituitary have been used for a long time to assess the secretory response of adenohypophysial tissue in the absence of hypothalamic or peripheral hormonal influences (Vale *et al.* 1972). Therefore, they may also provide a suitable *in vitro* model to study the proliferation features of anterior pituitary cell types and their reponse to growth factors. However, very little is known on their kinetics of replication. Early studies based on cell counting and nuclear incorporation of ^{3}H-thymidine showed that these monolayer cultures retained the capacity to proliferate up to three weeks (Rappay *et al.* 1973), with about 12% of cells replicating their DNA after eight days of plating (Rappay *et al.* 1980). In addition, *in vitro* studies using fragments of anterior pituitary demonstrated that an increasing number of cells entered DNA replication as the time of incubation with the radioactive analogue advanced; interestingly, an extremely low rate of proliferation was reported for fibroblasts (Mastro *et al.* 1969a). These findings suggested that differentiated cells of the anterior pituitary, very likely of epithelial origin, were able to synthesize DNA and divide also in *in vitro* conditions.

Similar data have been obtained in primary monolayer cultures using BrdU, where lactotrophs showed the highest analogue incorporation (Watanabe and Carbajo-Perez 1990). These results are in keeping with early observations that lactotrophs tend to dominate the culture with increasing time, whereas corticotrophs remain stable in number, somatotrophs and gonadotrophs progressively decline and thyrotrophs are extremely short-lived (Baker *et al.* 1973). Therefore, the pattern of replication and time-dependent distribution of the various hormone-producing, epithelial elements in monolayer cultures suggest that anterior pituitary cells retain *in vitro* a proliferation potential equivalent, to some extent, to that of the intact gland *in vivo*. Recently, using monolayer cultures and BrdU uptake (Fig. 1C-1K), we have been able to show that anterior pituitary cells from adult male rats diminish their proliferation rate after 7 days of plating as a consequence of a reduced transition of cells from the G0/G1- to the early S-phase. Reduction in G1/S transition is accompanied by

Fig. 1 - Light microscopic appearance of anterior pituitary cells from two-month-old male rats. A) Immunofluorescence distribution of thyrotrophs (red cells); B) double immunostaing (diaminobenzide and silver-intesified diaminobenzidine) of thyrotrophs (light brown cells) actively incorporating bromodeoxyuridine (BrdU) in their interphase nuclei (dark brown spots indicated by a black arrow) after *in vivo* administration of BrdU. Note the paucity of DNA-synthesizing with respect to quiescent thyrotrophs at this age. C-D) Differential interference contrast image of anterior pituitary cells grown in primary monolayer culture. Note striking differences in size and morphology of nuclei, reflecting heterogeneity of plated cell types. E-K) Immunofluorescence staining of asynchronously growing, cultured adenohypophysial cells, labeled with a monoclonal antibody to BrdU after *in vitro* administration of the synthetic analogue. BrdU-associated, intranuclear staining was recognized according to Humbert and Usson (1992) and classified in three distinct patterns following Nakayasu and Berezney (1989) as initial, intemediate and final steps of DNA synthesis: E-G) type I patterns, corresponding to early S-phase; H-J) type II patterns, corresponding to middle S-phase; K) type III pattern, corresponding to late S-phase.

an increase in size of BrdU-positive cells in the S-phase, suggesting that proliferating elements might exert contact inhibition, possibily mediated by paracrine mechanisms, on the committed cells in the quiescent pool, primarily acting within their G1-phase (Mosca and Toni 1994).

APPLICATION OF FLOW CYTOMETRY TO THE STUDY OF ANTERIOR PITUITARY CELL CYCLE KINETICS

Flow cytometry combined with immunocytochemistry has been applied to the sorting, purification and quantitation of different hormone-producing cell types from anterior pituitary glands of male and female adult rats (Hatfield and Hymer 1985, Wynick and Bloom 1990, Wynick *et al.* 1990a, Wynick *et al.* 1990b). In particular, using FSC and SSC signals three different groups of cells could be distinguished in terms of size and cytoplasmatic granularity, corresponding to three well defined light scatter peaks. In addition, a characteristic distribution of different cell types within each of these groups was obtained. Gonadotrophs and thyrotrophs were the biggest cells, followed by lactotrophs and somatotrophs whereas corticotrophs were the smallest epithelial elements. In contrast, somatotrophs had the highest granularity, followed by gonadotrophs and thyrotrophs whereas lactotrophs and corticotrophs contained a progressively smaller number of cytoplasmatic interfaces. Folliculo-stellate, endothelial and agranular cells displayed the smallest size and lowest granularity at all (Hatfield and Hymer 1986a, Hatfield and Hymer 1986b). A technical aspect, critical to quantify viable cells from dispersed anterior pituitaries, is the reported selective loss of gonadotrophs and thyrotrophs when these cells pass through the cytometer, possibly due to their higher fragility or adherence to the instrument tubing as a consequence of their big size (Hatfield and Hymer 1986a).

Similarly to freshly dispersed anterior pituitary cells, also cells from primary monolayer cultures have been reported to display three different light scatter peaks, corresponding to three different cellular populations with increasing size and granularity (Hatfield and Hymer 1986b). However, dramatic time-related effects of culture on light scatter distribution were noted. After 3 days of culture, irrespective of the presence or absence of serum in the culture medium, light scatter peaks with lower values tended to fuse, and between 8 and 12 days of culture a complete fusion of all the three light scatter peaks into one single dominant peak occured, suggesting that alterations to the structural integrity of the different cell types develop as the time of culture advances. This is in agreement with the dominance of a single cell type in the culture, possibly lactotrophs, as suggested by morphological studies (Baker *et al.* 1973, Watanabe and Carbajo-Perez 1990). However, Hatfield and Hymer (1986b) found that the number of lactotrophs from monolayer cultures was decreased after 1 week of plating, in both serum-free and serum-containing media. Since fibroblast growth becomes relevant after 12 days of plating in serum-containing media (Antakly *et al.* 1979), it is possible that, at least in part, the development of mesenchymal cells interferes with the growth of replicating epithelial cells; changes in shape and/or adherence of the latter to the growing substrate may equally play a role.

Recently, flow cytometry has been applied to the study of circadian fluctuations in the cell cycle of adult male anterior pituitary cells (Carbajo *et al.* 1992). A mitotic peak has been recognized at 20.00h, preceded by a peak of early S-phase cells at 11.00h and another peak of middle S-phase cells at 14.00h. In addition, whereas 0.6-1.7% of cells were found in the S-phase, only 0.1-0.3% were observed in the G2/M-phase. These data support a role for the light period as a regulator of cell replication *in vivo*, and confirm early cytological studies (Mastro *et al.* 1969a, Rappay *et al.* 1980, Carbajo-Perez and Watanabe 1990) indicating a low replication rate for the rat anterior pituitary tissue, irrespective of *in vivo* or *in vitro* methods of analysis. However, these data reveal also that only part of the replicating elements complete the cell cycle, suggesting that inhibitory factors control the progression of DNA-synthezising cells to their final mitotic state, irrespective of incoming photic stimuli.

Using a bivariate BrdU/DNA analysis and flow cytometry (Fig. 2A), we studied the cell cycle kinetics in primary cultures of two-month-old male rat anterior pituitary cells (Toni *et al.* 1991, Toni *et al.* 1992). We found that, despite the fact that the

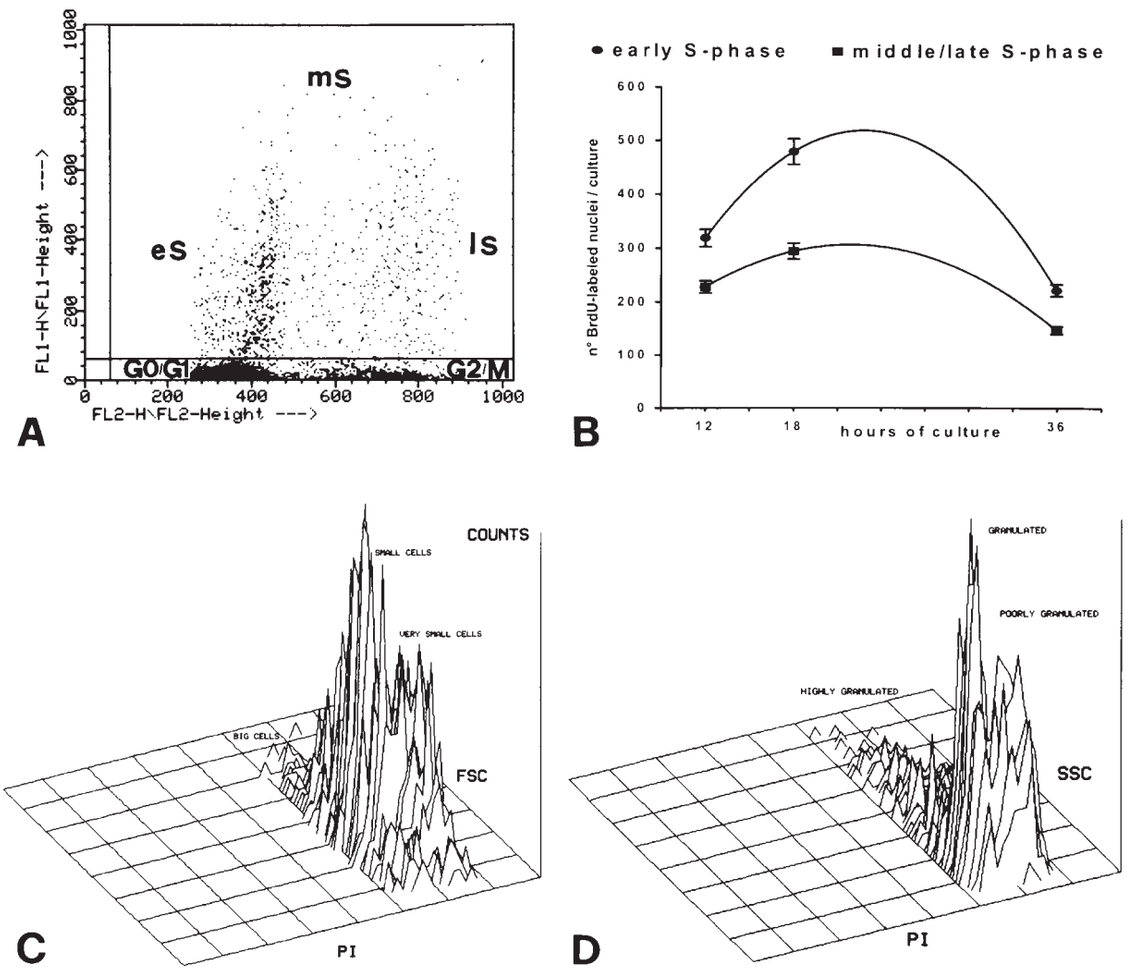

Fig. 2 - Flow cytometric analysis of cells from primary monolayer cultures of two-month-old male rat anterior pituitary. Viable cells were plated at 40,000/cm^2; after 72 hours (resting period), a culture medium change was made and cells were incubated with 15 µM BrdU/ 1.5 µM 5-fluoro-2-deoxyuridine for 2 h (end of incubation time between 10.00-11.00 a.m.). Cultures were then maintained for an additional 36 hours without any medium change. After fixation, cells were processed using fluorescein isothiocyanate-conjugated antibodies to BrdU, and studied by bivariate DNA/BrdU analysis. A) Tipical cytometric profile of diploid (G0/G1), tetraploid (G2/M) and DNA-synthesizing (early = eS, middle = mS, and late =lS S-phase) cells from a monolayer culture, 18 hours after the resting period; B) Polynomial approximation for the time course of S-phase during 36 hours of culture after the resting period. Note the presence of a periodic oscillation in the asynchronously, replicating population that covers an interval of around 24 hours. C-D) Light scatter profiles as studied by bivariate DNA/ Forward (FSC) or DNA/Side Scatter (SSC) analysis. Note that the culture is constituted by three different cell types in terms of size (FSC values) and granularity (SSC values), as shown in C) for FSC, 12 hours after the resting period and, in D) for SSC, 36 hours after the resting period.

cells retained their capacity to proliferate up to 7 days, a progressive, although not linear reduction in their replication rate occurred within this time frame. This reduction was primarily dependent on a diminished recruitment of cells from the diploid pool G0/G1, consistent with light microscopic observations and morphometric quantitation of DNA-synthesizing cells (Mosca and Toni 1994). Therefore, a reduced G1/S transition results as a major factor in the regulation of the proliferation potential of anterior pituitary cells *in vitro*, suggesting that this cell cycle check point might be a

target for inhibitory mechanisms involved in survival of DNA-synthesizing anterior pituitary cells. Interestingly, DNA synthesis and replication in the culture, corresponding to cells in S-phase, followed a pattern of around 24 hours, raising the possibility that anterior pituitary cells in primary culture undergo a periodic proliferation activity similar, although prolonged, to that observed *in vivo* during circadian oscillations (Fig. 2B).

Light scatter analysis, based on FSC and SSC parameters, revealed that the culture was constituted of three different cell types in terms of cell size and granularity (Fig. 2C, 2D), each maintaining its scatter profiles up to 7 days after plating. However, only two distinct cellular populations were recognized to initiate their DNA synthesis, i.e to enter early S-phase, the bigger and less granulated cells having the highest replication rate, which decreased to values comparable to the smaller and more granulated elements at the end of 7 days of culture (Mosca and Toni 1994). These data confirm the kinetics pattern observed with BrdU/DNA analysis, support heterogeneity of proliferating cells *in vitro,* and suggest that part of them may undergo a process of "degranulation" preceding their arrest at the G1/S border. Although the secretory nature of these two cellular populations remains to be elucidated, it is conceivable that the majority of the highly proliferating elements are lactotrophs, a possibility consistent with both previous flow cytometric studies, where prolactin-containing cells were observed to have a large size and paucity of intracellular interfaces (Hatfield and Hymer 1986a, Hatfield and Hymer 1986b), and with immunocytochemical *in vitro* studies (Baker *et al.* 1973, Watanabe and Carbajo-Perez 1990).

In contrast to some evidence (Harfield and Haymer 1986a), a critical aspect of our analysis has been the presence of cellular autofluorescence; it has been, in fact, necessary to run an isotype-matched, negative sample for anti-BrdU mAb fluorescence to recognize the brightest autofluorescent cells and set the proper compensation. Based on observations in different cellular systems, either a variable flavoprotein content or the presence of granules themselves (Del Castillo *et al.* 1989) might be responsible for the pituitary cell autofluorescence. Therefore, using cultured anterior pituitary cells, which are characterized by a low percentage of S-phase elements, accurate negative controls must be performed to avoid interference by autofluorescence during the flow cytometric analysis. In addition, pulse processing on the PI fluorescence signal may be required to eliminate doublets from the tetraploid region, based on the higher width of the fluorescence signal generated by cellular aggregates.

CONCLUSIONS

The rat anterior pituitary gland represents a suitable model for *in vivo* and *in vitro* studies of cell replication in a neuroendocrine tissue. It exhibits a low, cell type-, age- and sex-dependent replicating potential, and its proliferation activity seems to follow a circadian rhythm. Recently, flow cytometry has allowed purification of selective cell types and analysis of the effect of the light-dark cycle on cell replication, suggesting that this technique may be used to gain insights into the cell cycle steps involved in growth and differentiation of normal, anterior pituitary cells. Using primary cultures of rat anterior pituitary and a combination of flow cytometry, immunocytochemistry and morphometry, we have identified a peculiar pattern of proliferation activity *in vitro,* that changes during the time of culture, and exhibits a periodic behavior similar, to some extent, to that reported *in vivo,* raising the possibility that autocrine and/or paracrine influences regulate the replication rate of anterior pituitary cells in culture.

We conclude that the combination of primary monolayer cultures and flow cytometry is a promising approach to investigate the cell cycle kinetics of the anterior pituitary cells. It may also be applied to study the effect of growth factors on different anterior pituitary cell types as well as to characterize the growth features of tumoral adenohypophysial cells.

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