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Cell-cycle Dependent Differentiation of Friend Erythroleukemia Cells

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Abstract

Although Friend erythroleukemia (FL) cells have been largely believed to keep on proliferating after commitment for several generations before reaching the differentiated phenotype, there has been no certainity concerning the number of full cell cycles (CCs) necessary for these cells to be committed and differentiated in the presence of an inducer such as dimethylsulfoxide (DMSO). The present flow-cytometric analysis showed that FL cells, when treated simultaneously with thymidine and with DMSO, entered the culture growth cycle (CGC) with an S-phase, by conserving their potential to express hemoglobin (Hb). As a result of the double treatment, Hb was detected during the G_1-phase of the third CC, while its maximal intracellular concentration was reached during G_1 of the fourth CC (in the third CC DNA replication was significantly delayed by DMSO). Thus, one S was not sufficient for specific differentiation. The Hb increase occurred only when the inducer was present in the medium for a span of time including two subsequent S-phases. This, together with the measurement of the average length of the control FL cells lasting approximately 10 hrs, 240 min for G_1, 180 min for S, 140 min for G_2 and 40 min for M, showed that this basic finding was particularly supported by experiments which, at given points of the CGC, employed Colcemid to block mitosis or Aphidicolin (Aph) to block S.

The 745 A clone FL cells, originally derived from the spleen of a mice infected with a retrovirus complex [1,2], represented a useful model for in vitro differentiation studies [3]. They are similar to the erythroid progenitors blocked at the stage of pro-erythroblasts able to express Hb after treatment with DMSO [1-3]. This inducer-mediated differentiation was interpreted as a multi-step process [4], while two main events were recognized: the commitment to a terminal differentiation [5,6], and the acquisition of an erythroid-like phenotype [7]. The commitment to terminal differentiation was defined as the minimal time of inducer treatment requested by the cells to express the final phenotype [6]. In this framework, the question as to whether DNA synthesis is needed prior to the onset of globin mRNA transcription was rather diversified. On the one hand, there were data showing that induction of Hb synthesis can take place when DNA synthesis is inhibited [8]; on the other, it was claimed that at least one onset of DNA synthesis is required before the induction of Hb expression [2,9]. In addition, results were presented according to which the FL cells, arrested at a given stage by inhibitors of DNA synthesis or by isoleucine deprivation, fail to accumulate Hb, in contrast with the cells which are able to divide before becoming arrested [10]. Other investigators suggested that many FL cells accumulate globin mRNA over an appreciable span of time – more than one cell generation – after addition of an inducer [11] or relatively soon after induction [12]. Noteworthy was also the fact that commitment to differentiation and globin mRNA accumulation are coupled, while both events occur in G_1 after a pre-commitment phase of about 12 hrs [13]. This was in harmony with the suggestion that the site of hexamethylenebisacetamide leading to commitment is at a point of the G_1-phase prior to that of the CC block caused by deferoxamine or Aph [14]. Therefore, the question concerning the number of CCs needed for commitment and differentiation remained open.

The aim of this investigation was to accurately search, throughout the whole CGC, not only for the number of CCs required to induce Hb differentiation, but also for the precise phase of a single CC able to commit and ultimately express the complete quaternary structure of the respiratory protein. The hypothesis was that the FL cells cannot be committed simultaneously. One expected that more than one CC was necessary in order to reveal an initial Hb production. This was ascertained using Colcemid, to irreversibly block mitosis through inhibition of the microtubule assembly [15], and Aph, to block the S-phase through reversible inhibition of the DNA-polymerase (pol) system [16]. The analysis revealed that the FL cells, once synchronized through a double thymidine treatment [17,18], initiated their CGC with an S-phase, but did not alter their differentiating ability. Hb started to be detected during the G_1-phase of the third CC from inoculum, while its highest intracellular concentration was observed during the G_1-phase of the fourth CC. Thus, one S was indeed not

sufficient for specific differentiation. The Hb increase occurred only when the inducer was present in the medium for a time that included two subsequent S-phases.

Methodology

1. Cell growth. The FL cells were cultivated in humidified atmosphere of 5% CO_2, at 37 °C, in the RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 10 mM Hepes at pH 7.4. The CGC lasted approximately 4 days [19]. Cell differentiation was induced by addition of 1.5% DMSO (vol./vol.) to the medium [20]. Cell growth and mitotic index were evaluated by manual counting in a Neubauer hemocytometric chamber [18]. Cell viability was determined by the 'staining exclusion' test: $0.5x10^6$-$2.5x10^6$ cells were suspended in 0.5 ml phosphate buffer solution (PBS) before adding an equal volume of 0.4% Trypan blue prepared in physiological solution [21]. Following a 5-min incubation, the suspension was loaded onto the hemocytometric chamber to calculate the percentage of nonviable cells.
2. Cell synchronization. Cell synchronization was achieved through a double thymidine block, with a minor modification of the method described earlier [18]. The cells were seeded at the density of $0.3x10^6$/ml. Their partial synchronization was reached, in the presence of 2 mM thymidine, 10 hrs from inoculum. After washing, the suspension was maintained for 4 hrs in fresh medium without thymidine excess. The partially synchronized suspension was treated again for 10 hrs with 2 mM thymidine. At the end of this second thymidine treatment, the almost completely synchronized cells were washed and transferred, at the density of $0.3x10^6$-$0.4x10^6$/ml, into a medium containing 1.5% DMSO.
3. Arrest of cells in M and S phases. To obtain a block in metaphase, the cells were treated with 10 μg/ml Colcemid prepared in Hanks balanced salt solution [15]; to cause a block in S, they were treated with 0.2 μg/ml Aph prepared in DMSO.
4. Flow cytometric analysis. 0.5 $x10^6$ cells were used for the CC analysis. After washing in PBS, they were harvested and suspended in a staining solution containing 50 μg/ml propidium iodide (PI), 0.1% sodium citrate and 0.1% Triton X-100 [19,22]. The cells were left overnight at 4 °C in the dark before the analysis performed in an Epics V flow cytometer (Coulter, FL, USA). The Para-1 program (Coulter, FL, USA) was used for estimation of the cells distributed through the CC.
5. DNA labelling. Aliquots of suspension, containing $1x10^6$ cells, were withdrawn from the culture, washed with PBS, transferred into a medium containing 2 μCi/ml [^{3}H]thymidine (86.1 Ci/mmol) and incubated for 1 hr at 37 °C [19]. The DNA synthesis was evaluated on the basis of incorporation of the radionuclide into the TCA-precipitable material.
6. Hemoglobin determination. The Hb production was estimated through a spectrophotometric assay [13,23]. $2x10^6$-$4x10^6$ cells were washed with cold PBS and incubated for 15 min in a chilled solution containing 50 mM Tris-HCl at pH 7, 25 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 0.5% NP-40. After this incubation, the cells were centrifuged for 10 min at 10,000 g at 4 °C. The supernatant was used to determine the Hb content [24], using the extinction value of 1 OD at 414 nm for a 0.13 mg/ml Hb solution (the OD at 414 nm was checked by subtraction of that at 450 nm taken as blank).
7. Chemicals. The RPMI-1640 medium, the FCS and the other chemicals used for the cell culture were purchased from DASIT (Milano, Italy). The thymidine, DMSO, dithiothreitol, PI and Triton X-100 reagents came from Sigma-Aldrich (Milano, Italy). The Aph reagent was obtained from Alexis (San Diego, CA, USA), while the Karyo-Max Colcemid solution was purchased from Gibco Corporation (Sangiuliano Milanese, Italy). The [^{3}H]thymidine radionuclide came from Amersham (Piscataway, NJ, USA). The 0.45 μm filters (HAWP) were obtained from Millipore (Bedford, MA, USA). The Pico-Fluor 40 liquid scintillation cocktail was received from Packard (Groningen, The Netherlands).

Results

1. Cell synchronization and differentiation. A highly synchronized FL population, entering the S-phase (Tab. I), was seeded in the medium containing DMSO. The cell growth (Fig. 1a), the mitotic index (Fig. 1b), the rate of DNA synthesis (Fig. 1c) and the production of Hb per cell (Fig. 1d) were detected at regular time intervals through a CGC showing, in about two and a half days, 5 CCs and 4 cell division steps (the first CC was incomplete, without G_1). The first S, as determined by the incorporation of tritiated thymidine into genomic DNA, was completed 3 hrs from inoculum (Fig. 1c). After 3 more hrs, the mitotic index reached its maximal value (Fig. 1b). Although synchrony gradually decreased during the CGC (Fgs. 1a,b,c), the average length of the CCs in the differentiating culture was calculated to last roughly 10 hrs: 240 min for G_1, 180 min for S, 140 min for G_2 and 40 min for M. In such a framework, synchronization did not affect the cell differentiating potential, since an appreciable increase in Hb production started 26-28 hrs from inoculum (Fig. 1d). This timing was reasonably assigned to the early G_1 of the fourth CC (Fig. 1a).
Although all of the cells underwent the first mitosis, 75% of them were dividing after the second mitosis and 66% were dividing in each of the next M phases (Fig. 1a). This indicated that differentiation was characterized by a sequential recruitment of cells which were in a particular state. The rate of DNA synthesis showed that, after the first S, part of the cells entered the second S with a 2-hr delay, while in the third S two peaks of DNA synthesis were clearly originated (Fig. 1c). The first coincided with the expected S, whereas most of the cells divided 6 hrs later (Fig. 1a). During the fourth S, the rate of DNA synthesis was widely spread and very close to background values (not shown in figure 1c).
At the time corresponding to the initial Hb production, i. e. approximately 26-28 hrs from inoculum (Fig. 1d), synchronization was strikingly decreasing (Figs. 1b,c), while

more than 50% of cells showed a delay of S and M. From this one expected to find a number of cells in a prolonged G_1. The flow-cytometric analysis confirmed such an expectation, since 27 hrs from inoculum, 53.0% of the cells remained in S, while 36.0% were in G_1 and 3.2% were in G_2 (Tab. I).

A closer look at the distribution of phases during the third CC (Fig. 1a) revealed that, at the 22nd hr from inoculum, a higher percentage of cells was in S (Fig. 2a). Another increase in cell number occurred in G_2/M at the 26th hr from inoculum. This correlated well with the third CC (Figs. 1a,b). The fact that the percentage of S did not decrease too much with the increase of G_1 in the second part of the third CC reflected the origin of two peaks of DNA synthesis during this cycle (Fig. 1c). Such a result was further confirmed by using Aph from the beginning to the end of the third CC, i. e. starting 18 hrs from seeding in the presence of DMSO (Fig. 2b). In this case, Aph was able to maintain the cells in the G_1/S transition or in the initial S: 35% of the cells were in S in the first half of the third CC, while 60% of them remained in S in the second half (Fig. 2b).

At the 20th hr from inoculum, in the presence of DMSO alone, there was a 25% of cells in G_2/M (Fig. 2a). This did not correlate with any peak of DNA synthesis, beneath the preceding second CC. During this cycle, the cells could originate from a population that acquired a delayed S (5-6 hrs) or a longer G_2. Since during the CGC the delayed S-phases appeared to coincide with the increasing time required for cell divisions, one had to exclude the possibility that the lengthening of G_2 played a crucial role in differentiation. The percentage of cells in G_1 never went below 25%, in 10 hrs (Fig. 2a). In addition, the cells that presented a G_2/M delay of 5-6 hrs, leaving the second CC, were the same that reached the terminal G_1 in the third CC. Thus, during differentiation, the cell population appeared to be composed of three independently cycling groups characterized by different permanence in G_1 or by different entry into S: a larger group of cells entered S 3 hrs from mitosis (in this case G_1 lasted 3 hrs); another group of cells showed a 2-hr delay of S (in this case G_1 lasted 5 hrs); a third group of cells entered S 6 hrs later (in this case G_1 lasted 9 hrs).

2. Cell cycle-dependence of commitment to differentiation. For FL cells the DMSO-induced commitment to terminal differentiation was regarded as a multi-step process requiring at least 18 hrs from inoculum [3]. However, one had to specify the phase of the CC that favoured it. Taking advantage of the available synchronous system (Fig. 1), the cells were aliquoted in separate flasks and incubated in the presence of DMSO at different times, chosen on the basis of the lengths of the CC phases previously established. It turned out that, in order to obtain the initial Hb production, the synchronized cells required DMSO only during the first S and the first G_2 phases (Fig. 3); while the first S was probably necessary but not sufficient for commitment, the first G_2 was clearly crucial for the expression of the differentiated phenotype. The insignificant difference in Hb production between the first G_2 and the second G_1 suggested that the second G_1 was irrelevant for commitment.

3. The role of mitosis in differentiation. It was reported that mitosis would be an obligatory route to terminal differentiation and that many anticancer drugs acting on cell division would be able to induce it [25]. In a first set of experiments, this was checked through addition of Colcemid to unsynchronized FL cells grown in the absence or in the presence of DMSO (Fig. 4a). In the culture that was not treated with DMSO, the cell viability was reduced by 68%. At 24 hrs from inoculum the Hb production, induced by DMSO or Colcemid or by these two drugs together, was roughly the same (Fig. 4b). At 48 hrs from inoculum, while the Colcemid-treated culture showed a moderate increase in Hb production (when compared with the cultures that contained only DMSO or DMSO plus Colcemid), the amount of Hb per cell (in the case of addition of DMSO plus Colcemid) did not change (Fig. 4b). Therefore, Colcemid was able to induce Hb production, but this effect did not interfere with the DMSO-induced differentiation. In a second set of experiments, Colcemid was added to synchronized DMSO-treated FL cultures before the first mitosis. It led to a complete inhibition of cell multiplication and to a 70% decrease of cell viability (Fig. 5a). Figures 5a,b show that the Colcemid-induced Hb expression, at 28 hrs, was more than double that of the control culture which was treated with DMSO only (in this case the Hb production was similar to that measured for DMSO at 42 hrs). The loss of cell viability, at 42 hrs, was probably the reason for the lower Hb production detected at that time. Thus, Colcemid was able to induce an Hb production bypassing the requirement of several CCs needed in the case of DMSO, but it was unable to sustain the cell viability during differentiation even in the presence of DMSO. On such a basis, one could not consider Colcemid as a differentiation inducer, while the fact that it was able to block mitosis and progression of cells through subsequent CCs suggested that, starting from the second CC, it may be important in originating a survival signal required by the cells for terminal differentiation.

4. The role of DNA synthesis in differentiation. The role of DNA synthesis in the DMSO-induced terminal differentiation was still unclear, although a number of reports suggested that a prolonged permanence in G_1 or a transient inhibition of S was important for its development [3,25]. To shed some more light on the question, unsynchronized cells were seeded into a medium containing DMSO, while 18 hrs from inoculum (a sufficient length of time to reach commitment) a 0.2 μg/ml-concentration of Aph was added: in the presence of Aph, a complete inhibition of cell growth vs. a sustained cell viability was observed (Fig. 6a). At 42 or 66 hrs from inoculum, DMSO or Aph induced an equal Hb production (Fig. 6b). When these drugs were present together in the medium, a synergetic effect (leading to a maximal Hb production) could already be seen at 42 hrs (Fig. 6b). It should be mentioned that, since at 66 hrs from inoculum a 68% decrease of cell viability was observed, in the culture treated with Aph (Fig. 6a), this drug was not considered to be an inducer of differentiation. In contrast, the viability of the culture treated with Aph plus DMSO was maintained over 90% (Fig. 6a). This was indicative of terminal

differentiation; probably, the inhibition of DNA synthesis, due to Aph, may be able to anticipate the terminal differentiation. The step-up process of terminal differentiation, obtained with the synchronized differentiating system, was thus a test to establish whether the addition of Aph, after commitment, could increase the percentage of cells with inhibited DNA synthesis and, through such an increase, to anticipate the terminal differentiation.

Discussion

On the basis of the fact that, in growing DMSO-differentiating FL cells, Hb is intensively exhibited 48 to 96 hrs from inoculum [19], the idea of this study was to shed some new light on three interrelated main questions concerning (i) the number of the CCs needed to reach the Hb production, (ii) the timing of the pre- and commitment conditions preceding its actual appearance and (iii) the phase of the CC, G_1 or G_2, favouring its full expression.
As for the first point, the data showed that, in the presence of DMSO, the initial shorter G_1-deprived CC (Fig. 1a) led to the commitment in G_2 of 25% of cells (Fig. 2a), while the Hb production – apparently observed in G_1 of the fourth CC (Fig. 1a) – occurred de facto in G_1 of the third CC, in harmony with the CC phase distribution in the presence of 'no more cycling' cells in this third CC (Figs. 2a,b). In other words, only two CCs were necessary to achieve the differentiating phenotype. In this time-lapse the role of the second CC was intriguing. Colcemid, generally known to block mitosis, was also able to induce the Hb production in the case of FL cells (Figs. 4 and 5). However, this drug was unable to sustain their viability: when it was added to the medium before the first mitosis achieved by the synchronized differentiating culture, viability of the cells producing Hb decreased with time even in the presence of DMSO. Through a block of mitosis, the CC progression was inhibited. Thus, the second CC would have been necessary to express a surviving signal, specific for erythroid lineage.
As for the second point, the attention is to be focused on the role of the S-phase as a whole. Differentiation in any growing unsynchronized FL culture was known to be a multi-step process characterized by a sequential recruitment of cells which progressively enter a particular stage [4]. In the synchronized culture here considered, differentiation was instead reached after a relatively long time (Fig. 1a) characterized by an increasing delay and a gradual inhibition of the succeeding S-phases (Fig. 1c). Moreover Aph, when added to the differentiating synchronized cells before the second and the third S-phases, was able to delay the corresponding cell divisions by 10 hrs (Fig. 6). This indicated that, at least at the concentration used in the present work, the drug caused only a reversible G_1-block**.** For this reason, one may think that it likely synergized DMSO for recruitment of all cells being in a particular state. Such a state, in turn, favoured commitment. However, the initial Hb production in synchronized cells was not affected by Aph (Fig. 5b).
Without forgetting, of course, that the mechanism of action of Aph might also interfere with that of DMSO, this suggested that only the not yet DMSO-committed cells were sensitive to its synergetic effect on differentiation, while the lengthening of G_1 induced by the same Aph was actually part of a pre-commitment state.
The third point can be discussed in connection with the effect of DMSO on DNA-replication. The incorporation of tritiated thymidine into the FL genomic DNA showed three separate pools of cells (Figs. 1a,c): one with regular S (present during the first CC and at the beginning of the second and third CCs), another with 2-hr delayed S (present in the second CC), and a third with 6-hr delayed S (present in the third CC). The delay of S, obviously, always implied lengthening of G_1, since G_2 and M did not exceed their regular timing. This suggested indeed a direct correlation between the lengthening of G_1 and the peculiarities of the terminal differentiation. The fact that here, in the FL cells, Hb was ultimately produced in G_1 is consistent, on the one hand, with the data provided by Pragnell et al. [13] and Kijokawa et al. [14] and, on the other, with the background knowledge suggesting that, for many mammalian cells, the bulk protein is produced for the most part in G_1 and G_2 [26], while few proteins are made in S, for instance pols [16] and histones [27], or only exceptionally in M [28].
The present work, essentially suggesting that in growing synchronized FL cells an inducer of differentiation like DMSO causes a sequence of events (at least two full CCs) before the exhibition of Hb as a complete protein, acquires significance: this is not only in relation to the studies concerning the role of the CC regulation in differentiation, in general, or to those correlating apoptosis and cancer [29], but also to investigations on the regulation of gene expression when this is inversely correlated, for instance, with a specific promoter methylation [30], this being a matter of great interest in Genome Biology and particularly in Ontology.

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Table I. Distribution of the cell cycle phases in the synchronized population of FL cells entering the S-phase. The culture released from the second thymidine treatment was transferred into a fresh medium without thymidine (Fig. 1a) and analysed at time intervals from inoculum.

	DISTRIBUTION (in %)*		
Hrs	Cells in G_1	Cells in S	Cells in G_2-M
1	11.0	86.0	2.5
2	8.9	87.0	2.6
3	21.0	66.0	8.6
5	10.0	26.0	62.3
8	68.2	2.7	7.5
27	36.0	53.0	3.2
29	22.0	65.0	12.0
31	20.0	55.0	14.0

**The calculation was performed using a constant number of cells ($0.5x10^6$). The data represent the mean of three experiments.*

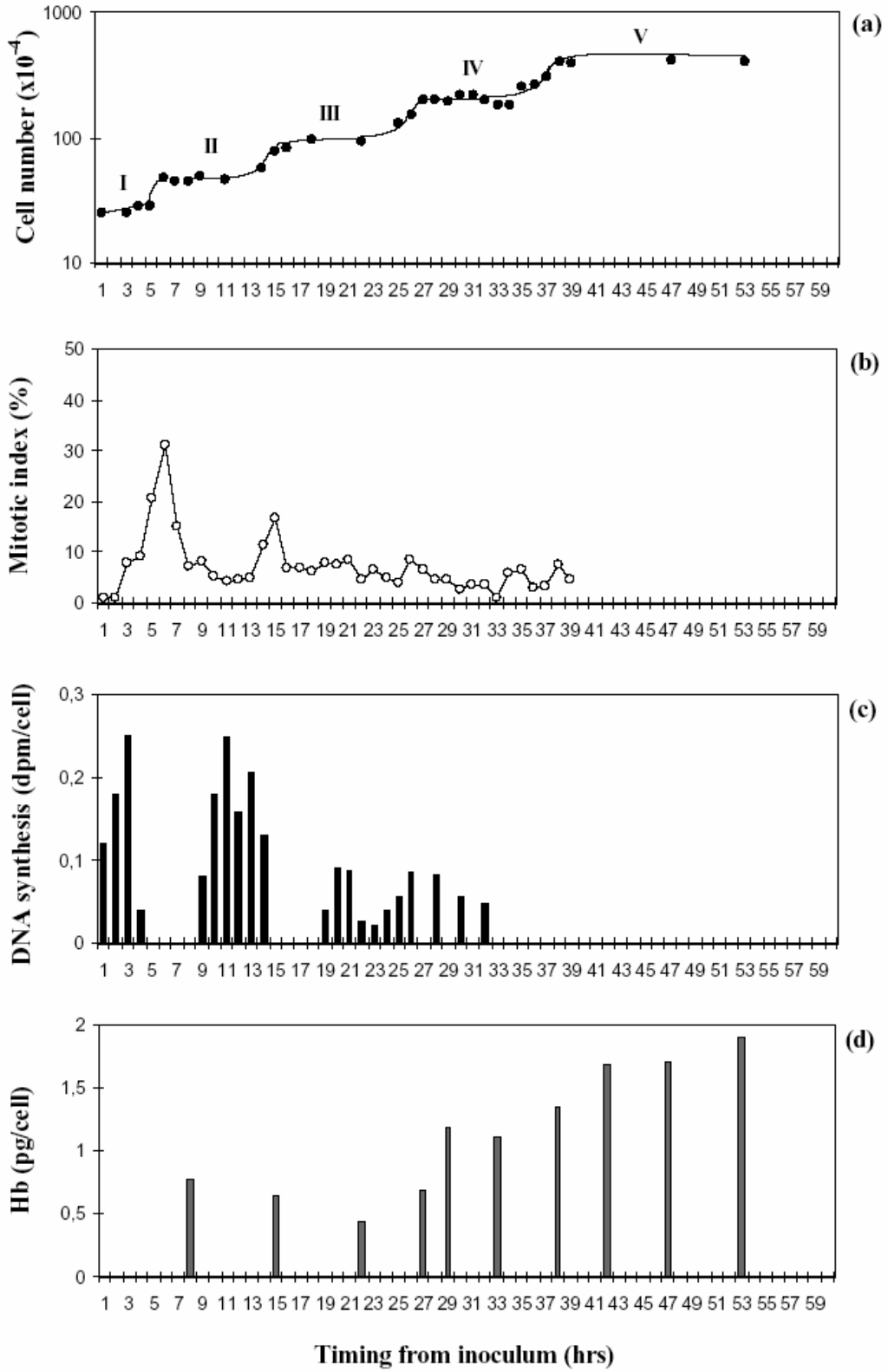

Fig. 1. Induction of Hb differentiation in synchronized FL cells. After removal of the second thymidine excess, the synchronized cells were reversed at the density of 0.3x10^6/ml into fresh RPMI-1640 medium containing 1.5% DMSO and left to grow in it for 60 hrs. (a) Density of the cell population. (b) Percentage of the cells in division. (c) Rate of the [^{3}H]thymidine incorporation per cell (2 μCi/1.0x10^6 cells/ml for 1 hr at 37 °C). (d) Hb concentration per cell. The curve of cell proliferation (a) and that of cell division (b) were repeated five times; the [^{3}H]thymidine uptake by cells (c) and the Hb concentration per cell (d) represented the mean of two experiments.

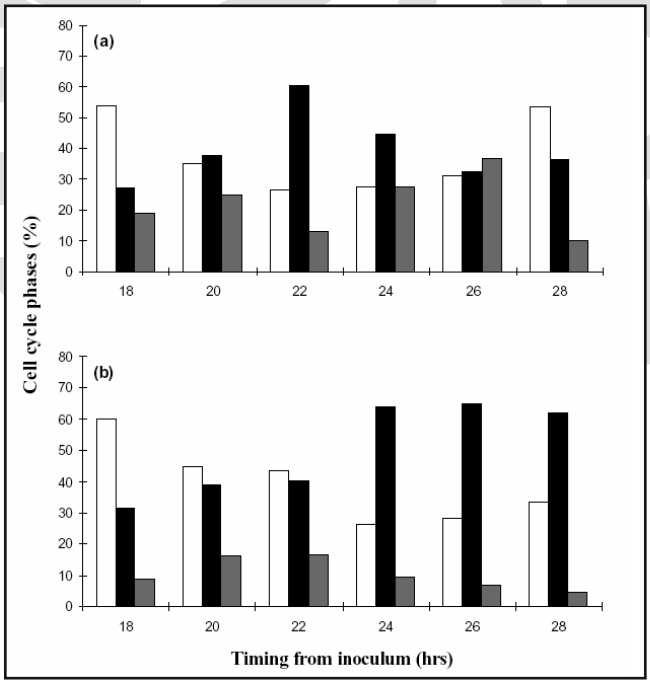

Fig. 2. Flow-cytometric analysis during the third CC of synchronously growing FL cells induced to differentiate Hb through DMSO. Aliquotes of 0.5x10^6 cells were withdrawn at various times from inoculum (Fig. 1a) and incubated overnight in 0.5 ml of staining solution. (a) Cells synchronized with the double thymidine block and grown in the presence of 1.5% DMSO. (b) Cells synchronized with the double thymidine block and grown in the presence of 1.5% DMSO plus 0.2 μg/ml Aph added before the third S-phase (18th hr from inoculum). G_1, white columns; S, black columns; G_2/M, gray columns. The experiment was repeated twice.

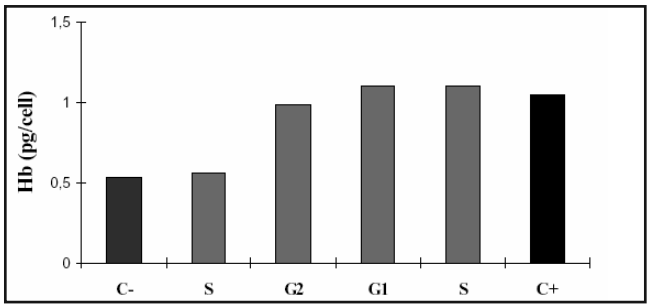

Fig. 3. Commitment of the Hb differentiation in FL cells treated with DMSO. Aliquotes of synchronized cells entering S were seeded in a number of flasks at the same density (0.5x10^6 cells/ml) but incubated with 1.5% DMSO for different times. These times covered S or G_2 of the first CC and G1 or S of the second CC (Fig. 1a). At the end of an incubation, each cell sample was washed, seeded in the absence of DMSO and left to grow for 28 hrs. At this time, the Hb production was monitored: (C-) control unexposed cells; (C+) cells exposed to 1.5% DMSO. The experiment was repeated three times.

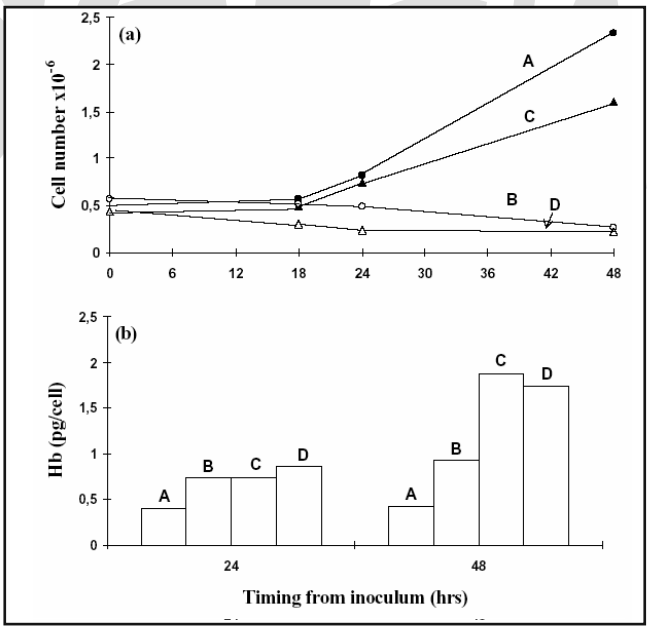

Fig. 4. Influence of Colcemid on the Hb production in unsynchronized FL cells. (a) The cultures were inoculated at the density of 0.5x10^6 cells/ml in the RPMI-1640 medium and left to grow in it for 48 hrs. (b) The amount of Hb per cell was monitored 24 and 48 hrs from inoculum. (A) Cells grown in the absence of Colcemid or DMSO; (B) cells grown in the presence of 0.1 μg/ml Colcemid; (C) cells grown in the presence of 1.5% DMSO; (D) cells grown in the presence of 0.1 μg/ml Colcemid plus 1.5% DMSO. The experiment was repeated twice.

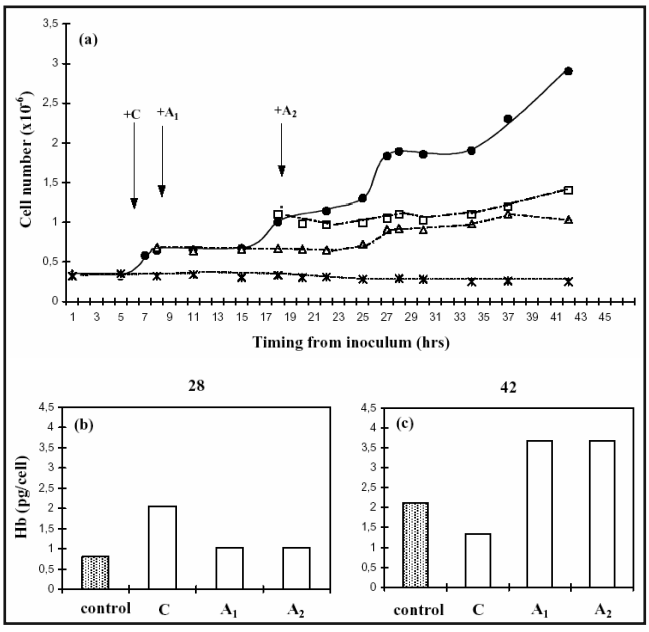

Fig. 5. Influence of Colcemid or Aph on the Hb production in synchronized FL cells. The cells entering the S phase were inoculated at a density of 0.3x10^6/ml in the RPMI-1640 medium containing 1.5% DMSO and left to grow in it for 48 hrs (panel a). In a first experiment, before the first M-phase, a 0.1 μg/ml-concentration of Colcemid (+C) was added to the medium (stars in panel a and white columns in panels b and c). In a second experiment, before the second S-phase, a 0.2 μg/ml-concentration of Aph (+A1) was added to the medium (triangles in panel a and white columns in panels b and c). In a third experiment, before the third S-phase, a 0.2 μg/ml-concentration of Aph (+A2) was added again to the medium (squares in panel a and white columns in panels b and c). The production of Hb per cell was estimated 28 hrs (panel b) and 42 hrs (panel c) from inoculum. The gray columns in b and c, indicated as control, showed the Hb production by the cells treated with 1.5% DMSO only. The data represent the mean of three repetitions.

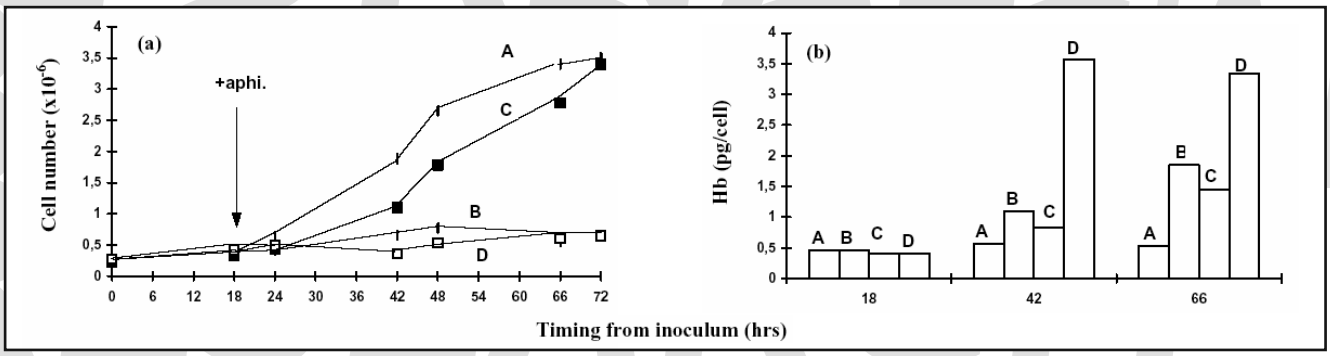

Fig. 6. Addition of Aph to unsynchronized FL cells after commitment. (a) The cells were inoculated at the density of 0.3x10^6/ml in the RPMI-1640 medium, enriched with 10% foetal calf serum and 1.5% DMSO, and allowed to grow for 72 hrs. (b) Hb content per cell at 18, 42 and 66 hrs from inoculum (a value of 3.6 pg of Hb per cell, obtained from several cultures treated for 96 hrs with DMSO, was taken as "maximal"). (A) Control cells; (B) cells grown in the presence of 0.2 μg/ml Aph added to the culture 18 hrs from its inoculum; (C) cells grown in the presence of 1.5% DMSO; (D) cells seeded in a medium containing 1.5% DMSO enriched with 0.2 μg/ml Aph 18 hrs from inoculum. The experiment was repeated twice.