Originals

Cell cycle and gene expression in the insulin producing pancreatic cell line β TC1

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Summary. The pancreatic cell line β TC1, established from insulinomas of transgenic mice carrying a hybrid insulin-promoted large T antigen gene, has retained several characteristics of normal cells, including the insulin content and inducibility of insulin secreting by glucose. We show here that the growth of β TC1 cells is arrested in low serum-concentration medium. Cells exposed for three days to 0.25% fetal calf serum ceased to incorporate [3 H] thymidine but were still able to resume the cell division cycle upon addition of serum. In this cell line, we have determined by cytofluorometry the cell cycle kinetic parameters to be of 21 h, 10 h 30 min and 12 h for the G₁, S and G₂/M phases, respectively. Quiescent β TC1 cells constitutively expressed the protooncogene *c-jun* that codes

for the transcriptional factor AP1, as well as *cdc2*, another cell cycle-related gene. A large transient increase in the expression of the *c-fos* gene was obtained rapidly, 30 min after addition of serum and a similar increase in *c-jun* expression after one hour. Expression of the *cdc2* gene was also enhanced to a lesser extent. The same effects were also observed in the presence of cycloheximide, thus proving that the expression of these three genes is directly stimulated by serum growth factors. Consequently, quiescent β TC1 cells provide a good model for studying the short- and long-term effects of growth factors on Beta-cell proliferation.

Key words: Beta cell growth, cell cycle, oncogenes.

It is of crucial importance to understand the mechanisms which control the Beta-cell mass, since the insulin deficiency observed in Type 1 (insulin-dependent) diabetes mellitus is associated with the destruction of insulin-secreting cells and little is known about the mechanism of Beta-cell renewal. Most of the studies on G₁ events and regulation of cell proliferation have been carried out with fibroblasts and with haematopoietic cells [1]. The results show that the regulation of cell proliferation differs according to the cell type. In the pioneering work of Swenne [2], the analysis of the Beta-cell cycle was approached using fetal islets cultured in vitro. Fetal Beta cells were shown to have a high proliferative capacity [2]. Those results and the finding that Beta cells can be induced to re-enter the cell cycle by appropriate environmental signals [3] represented a considerable advance in the understanding of Beta-cell function. The recent development of insulin-producing cell lines such as those obtained from hamster insulinoma tumours (HIT cells) [4], from rat insulinoma (RIN cells) [5], rat transplantable islet cell tumour (CRI cells) [6] or SV40 T-Antigen transgenic mouse (IgSV195) [7] provides remarkable new models for the study of Beta-cell regulation. We have used the β TC1 cell line recently established by Efrat et al. [8] from insulinomas of transgenic mice carrying a hybrid insulin-gene: large T antigen of simian virus 40 under the control of the

rat insulin II promoter; this cell line retains morphological characteristics of pancreatic Beta cells. β TC1 cells were shown to synthesize and release insulin [8] and to stimulate DNA synthesis upon glucose stimulation [9]. In this work we have determined the conditions under which β TC1 cells become quiescent through depletion of serum and the subsequent resumption of the cell division cycle upon external stimulation.

Materials and methods

Materials

Dulbecco's modified minimum essential medium (DMEM) was obtained from Intermed (Noisy le Grand, France). Fetal calf serum (FCS) was from Boehringer Mannheim (Meylan, France). [3 H] thymidine was purchased from Amersham (Les Ulis, France). Platelet-derived growth factor was obtained from Collaborative Research Inc. (Bedford, MA, USA). Other chemicals were of reagent grade.

Cell culture

β TC1 cells were kindly supplied by D. Hanahan and routinely cultured in DMEM medium (25 mmol/l of glucose) supplemented with 8% FCS, 100 U/ml penicillin and 100 mg/ml streptomycin. They were plated at about 10% confluence and passed once a week when

Fig. 1. Arrest of β TC1 cell growth in medium containing a low serum concentration. Serum deprivation was allowed to proceed for 3 or 4 days in 0.25% fetal calf serum Dulbecco's modified minimum essential medium. Cells were then submitted to serum stimulation (8% fetal calf serum, hatched bars) or medium change (open bars) in the presence of [3 H] thymidine (1 μ Ci/ml) for the following 24 h. The incorporated radioactivity was determined as described in Materials and methods. Results are means and SEM of triplicate determinations of a representative experiment

they reached about 50% confluence, as recommended by Efrat et al. [8]. The medium was changed every two to three days. We have ensured that β TC1 cells retain their ability to respond to glucose by insulin secretion [8]. During all experiments the glucose concentration was kept to 25 mmol/l.

Measurement of the rate of DNA synthesis

For studies of mitogenic effects, cells were seeded in Corning 24-well dishes (180 000 cells/well in 1 ml volume) in medium supplemented with 8% FCS. The cells were washed twice with 1 ml of serum-free medium 24 h after seeding. The latter was replaced with a medium containing 0.25% fetal calf serum for three days, unless otherwise stated. After serum deprivation, the cells were stimulated by serum or mitogenic factors in the presence of [3 H] thymidine (1 μ Ci) for the following 24 h. Incorporation of the labelled precursor into DNA was terminated by adding three drops of 1 mol/l ascorbic acid [10]. Cells were washed twice with cold phosphate-buffered saline (PBS), twice with cold 5% trichloroacetic acid (TCA), and solubilized in 0.3 ml of 0.1 mol/l NaOH, 0.1% SDS. An aliquot (0.25 ml) of the solubilized cells was mixed with 5 ml of Aqualyte Scintillation Liquid (JT Baker B. V., Deventer, The Netherlands) and counted in an LKB beta-spectrometer.

Analysis of RNA

For RNA extraction, $25 \cdot 10^6$ cells were seeded in 100 mm Petri dishes ($5 \cdot 10^6$ cells/dish). Twenty four hours after seeding, the cells were washed twice with serum-free medium and rendered quiescent by incubation in 0.25% FCS-containing medium for three days. After the treatment described in the figure legends, cells were chilled on ice, washed once with cold PBS and total RNA was isolated using the Li-Cl/urea precipitation procedure described by Auffray and Rougeon [11]. Twenty micrograms of total RNA was denatured in 2.2 mol/l formaldehyde for 10 min at 60°C, fractionated by electrophoresis in formaldehyde 2.2 mol/l agarose 1% gel, transferred onto a nitrocellulose filter, fixed by heating at 80°C and hybridized overnight at 42°C with 32 P-labelled probes (labelled by Multiprime, (Amersham) to a specific activity of $2\text{--}4 \cdot 10^9$ dpm/ μ g) in $5 \times$ SSPE,

$5 \times$ Denhardt's, 0.1% SDS and 50% formamide, according to Maniatis et al. [12]. The blots were then washed twice in $2 \times$ SSC, 0.1% SDS for 15 min at room temperature, for 1 h at 45°C in the same buffer and then for 30 min in $0.1 \times$ SSC, 0.1% SDS at 45°C. Blots were exposed to X-ray films (Kodak XAR5) with intensifying screens (Dupont Cronex type Quanta III, Euromedica, Paris, France) at -80°C . Exposure time varied from overnight to five days.

Probes

The probes were rat α -tubulin cDNA [13]; Bgl II-Pvu II fragment of FBJ provirus (*v-fos*) [14]; 3rd exon of human *c-myc* probe [15]; mouse *cdc2* cDNA (kindly provided by P.Nurse) and RJA1 clone corresponding to mouse *c-jun* cDNA [16].

Flow cytometry analysis (FCM)

Exponentially growing cells ($10 \cdot 10^6$ cells) were stained in the culture dish for 1 h with 5 μ g/ml Hoechst 33342 (Sigma, La Verpillière, France) at 37°C in standard culture medium [17]. After washing in PBS buffer containing 1.5 mmol/l EDTA, cells were harvested with trypsin-EDTA, washed again with the PBS-EDTA buffer and suspended at a density of 10^6 cells per ml in DMEM medium containing 2% FCS and 1.5 mmol/l EDTA. Immediately prior to FCM analysis cells were filtered through a 37 μ m nylon monofilament mesh filter. The fluorescence of the stained cells was monitored on a FACS 440 (Becton Dickinson, Mountain View, CA, USA) interfaced to a MCA 3000 computer (Bruker, Wissembourg, France). The excitation was with the 365 nm line of a Spectra Physics model 20-20 argon ion laser, and emission was measured using a 450 nm long pass filter and a 450 nm Oriel band pass filter. Three synchronisations of exponentially growing cells were performed in the cell sorter and two populations were sorted in each synchronisation: 1. cells in G_1 and in S, 2. cells in G_1 and in G_2/M and 3. cells in S and in G_2/M . The three fluorescence profiles obtained were strictly superimposable to each other. They were analysed according to the Fox model [18]. The sorted cells were collected in 20% FCS, DMEM medium under sterile conditions and replated in 35 mm Petri dishes (10^5 cells/dish). The replated cells were again stained with Hoechst 33342 for 1 h in the culture dish, collected and filtered as described above before FCM analysis. A control calibration histogram with exponentially growing cells was performed 24 h after the first synchronization at this lower cellular density (5 to $10 \cdot 10^4$ cells/ml); this histogram was perfectly superimposable with those obtained in the three synchronizations (not shown). FCM analysis was performed 7, 10, 11, 14, 18 and 21 h after replating the cells synchronized in G_1 ; 7, 9 and 10 h 30 min for the cells synchronized in the S phase; 6, 7, 10 and 12 h for the cells synchronized in G_2/M . Only three representative points are presented in the Results.

Results

Arrest of β TC1 cell growth in medium containing low serum concentrations

Serum deprivation for two to three days was required to arrest β TC1 cell growth (Fig. 1). Cells exposed for three days to 0.25% FCS ceased to incorporate [3 H] thymidine but could resume their cell division cycle during the following 24 h upon serum addition. Incubating the cells for an additional day in the serum-depleted medium led to a dramatic decrease in [3 H] thymidine incorporation for the following 24 h, in control cells as well as in serum stimulated cells (Fig. 1). Cell viability was $>90\%$ in the serum-

Fig. 2. Effect of serum on DNA synthesis in β TC1 cells. Cells were rendered quiescent by a 3-day incubation in low serum containing medium. The medium was then replaced (zero time) with an 8% fetal calf serum Dulbecco's modified minimum essential medium (FCS DMEM) (black circles) or for control cells, with a 0.25% FCS DMEM medium (open triangles). After 14 h, [3 H] thymidine pulses (2 μ Ci/ml) of 2 h duration were applied every 3 h. The incorporated radioactivity was determined as described in Materials and methods. The data shown are means of duplicate values and are represented at the mean times of thymidine pulse application

Fig. 3. β TC1 cell cycle kinetics. Exponentially growing β TC1 cells were synchronized in a cell sorter in the various cycle phases as described in Materials and methods and replated for various periods of time. Only three representative points are presented for each phase of the cell cycle. The distribution of the fluorescence of the cell population shown by black lines corresponds to the replated cells; the initial distribution of the asynchronous population is presented in grey. **Left:** G₁ phase, 7, 10 and 21 h from top to bottom. **Middle:** S phase, 7, 9 and 10 h 30 min from top to bottom. **Right:** G₂/M phase, 6, 7 and 12 h from top to bottom

depleted medium until the end of the fourth day of incubation, i.e. three days of deprivation plus one day in the presence of [3 H] thymidine (data not shown). Cells starved of serum for three days were thus defined as quiescent cells in the following experiments.

The time dependence of [3 H] thymidine incorporation is shown in Figure 2. The stimulation of [3 H] thymidine incorporation was already detected 14 h after serum addition, maximal stimulation being observed at 23 h. The incorporation decreased afterwards. In the control cells the [3 H] thymidine incorporation remained very low throughout the experiment (Fig. 2). As described by Swenne, the width of the first DNA synthetic wave (measured at a height of 50% of the peak value), can be used as an index of the mean S phase duration [2]. In β TC1 cells the length of the S phase can be roughly estimated to about 10 h.

Kinetics of the β TC1 cell cycle

The duration of each phase of the β TC1 cell cycle was determined in two steps by cytofluorometry of exponentially growing cells (Fig. 3). In the first step, the selected populations were synchronized in the different phases of the cell cycle and then replated (see Materials and methods). In the second step, the replated cells were analysed at different time intervals after replating. The duration of a given phase was defined as the minimum time for all the cells to leave this phase. The G₁ phase was the longest phase of the β TC1 cell cycle (Fig. 3). Seven hours after replating, only a small number of cells had moved from the G₁ to the S channels (Fig. 3, G₁, top) and after 10 h about 50% of the cells were still in G₁ (Fig. 3, G₁, middle). All the cells had left the G₁ phase 21 h after replating (Fig. 3, G₁, bottom). The S phase was more rapid: as soon as seven hours after replating, about one-third of the population had shifted to the G₂/M channels (Fig. 3, S, top). The S phase was completed after 10 h 30 min (Fig. 3, S, bottom), confirming the length of 10 h estimated in the [3 H] thymidine experiment. Six hours after reseeding, nearly all cells synchronized in G₂/M were remaining in their initial channels (Fig. 3, G₂/M, top) but after one additional hour, a large proportion of cells had left the G₂/M channels (Fig. 3, G₂/M, middle). Twelve hours after replating, all the cells had left the G₂/M channels and entered the next G₁ phase (Fig. 3, G₂/M, bottom). We conclude that the lengths of the different phases of the β TC1 cell cycle are 21 h, 10 h 30 min and 12 h for the G₁, S and G₂/M phases, respectively. The complete generation time of the β TC1 cells is therefore 42 to 43 h, which is consistent with the doubling time of 45 h calculated from typical growth curves (data not shown).

Dispensability of platelet growth factors for β TC1 cell growth

In order to determine the involvement of platelet factors in the resumption of the β TC1 cell cycle, cells were rendered quiescent by serum depletion, and then submitted to various concentrations of human adult platelet-poor plasma or serum. Figure 4A shows that both the serum and plasma were highly mitogenic in β TC1 cells. Their effects were similar throughout the concentration range studied (0.25 to 8%). Furthermore, purified platelet-derived growth factor (PDGF, 1 U/ml), in the presence of

Fig. 4A, B. Dispensability of platelet growth factors for β TC1 cell growth. Cells were depleted of serum for 3 days as described in Materials and methods. The medium was replaced with serum-free medium, with which the cells were washed twice. **Panel A:** serum-free medium (open bars) or various concentrations of human platelet-poor plasma (black bars) or of the corresponding human serum (hatched bars), were then added simultaneously with [3 H] thymidine (1 μ Ci/ml) for the following 24 h. **Panel B:** cells were submitted to serum-free medium or 0.25% fetal calf serum (FCS) (open bars), 0.25% FCS + 1 U/ml platelet derived growth factor (PDGF) (hatched bars) or 8% FCS (black bars) in the presence of [3 H] thymidine for 24 h. The incorporated radioactivity was determined as described in Materials and methods. Data shown are means and SEM of triplicate determinations

Fig. 5. Expression of the cell cycle-related genes. Cells were rendered quiescent as described in Materials and methods. Total RNA was extracted, immediately, after 30 or 60 min of stimulation by 8% fetal calf serum. Twenty μ g of total RNA was analysed in Northern blots and hybridized with [32 P]-labelled probes, as described in Materials and methods. Sizes of the mRNAs: *c-fos* 2.2 kb [14], *c-jun* 2.7 kb and 3.2 kb [16], *cdc2* 1.6 kb, and α -tubulin 1.8 kb [13]. Exposure time was overnight for *c-jun*, *cdc2* and α -tubulin, and 24 h for *c-fos*

0.25% FCS, did not induce the quiescent cells to enter the cell cycle (Fig. 4B). These results indicate that neither PDGF nor other platelet factors seem to be necessary for β TC1 cell proliferation. Furthermore the enhancement of [3 H] thymidine incorporation either with human plasma or serum vs fetal calf serum, indicates that other factors or nutrients advantageous for β TC1 cell growth, might be present in human sera.

Expression of cell cycle-related genes

The initiation of the cell division cycle is correlated to the enhanced expression of specific genes [1]. The time course for the stimulation of these cell cycle-related genes was analysed in the early phase of serum induction. RNA from quiescent β TC1 cells was extracted immediately (lane 0), 30 min or 1 h after addition of serum (Fig. 5). In the quiescent β TC1 cells, the *c-jun* mRNA was already detectable and *cdc2* mRNA was also constitutively expressed. While no *c-fos* mRNA was detected in quiescent cells, a strong and fast induction of *c-fos* gene expression occurred in the serum-pulsed cells. Maximal levels of this mRNA were obtained after 30 min of stimulation and decreased thereafter. *C-jun* expression was enhanced at 30 min and then remained approximately constant during the first hour. After more than 3 h in the presence of serum, *c-jun* mRNA levels decreased to basal levels (not shown). Only a slight increase in the *cdc2* mRNA level was observed after 1 h of treatment with serum. No change in the expression of α -tubulin was detected under those conditions, in agreement with the stable expression of α -tubulin gene observed in other systems during the early phases of the cell cycle [19]. The *c-myc* mRNA could not be detected with serum stimulations varying from 30 min to several hours (not shown).

Cycloheximide has been reported to superinduce several early genes by stabilizing the corresponding mRNAs and preventing the down-regulation of transcription by the protein products [20, 21]. Furthermore, cycloheximide allows the analysis of the direct effects of serum on cell cycle-related gene expression. We therefore treated quiescent β TC1 cells for 2 h with cycloheximide (1 μ g/ml) with nothing (lane 1) or with 8% serum (lane 2). The cellular RNAs were hybridized with the radioactive probes as shown in Figure 6. The cell cycle-related genes *c-fos*, *c-jun* and *cdc2* were expressed at a low level in the cycloheximide-treated cells in the absence of serum. The *c-myc* mRNA was hardly detectable. In addition, serum induced the accumulation of mRNA corresponding to the cell cycle-related genes *c-fos* and *c-jun* (lane 2). Superinduction by cycloheximide was also observed for the *cdc2* mRNA, and was more unexpected for a gene known to be induced in the cell cycle much later than *c-fos* and *c-jun* [22]. Only a very slight induction of the *c-myc* mRNA occurred in the cells treated with cycloheximide and serum (lane 2).

Discussion

The data presented in this report were obtained with pancreatic β TC1 cells. This cell line was established by Efrat [8] from insulinomas derived from transgenic mice carrying hybrid insulin-promoted SV40 large T antigen. These cells possess characteristics of differentiated normal Beta cells [8]. Although their insulin content is somewhat lower than that of Beta cells in cultured mouse islets, they have retained the glucose-induced insulin secretion and express high steady state levels of insulin mRNA [8, 9]. We have established the conditions of serum depletion

Fig. 6. Expression of the cell cycle-related genes in the presence of cycloheximide. Quiescent cells left for 3 days in 0.25% fetal calf serum (FCS) medium were treated for 2 h with 1 μ g/ml cycloheximide (a concentration sufficient to inhibit more than 90% of protein synthesis) either alone (lane 1) or in the presence of 8% FCS (lane 2). Twenty micrograms samples of total RNA analysed by Northern blots was hybridized with the indicated radiolabelled probes, as described in Materials and methods. The exposure time was 24 h, except for the *c-myc* probe: 5 days

allowing the cells to enter quiescence: when cultured for three days in medium containing 0.25% FCS, the β TC1 cells cease to incorporate [3 H] thymidine and can be recruited into the cell division cycle upon the addition of serum. This provided a model permitting the analysis of the early events associated with β TC1 cell growth, including the expression of cell cycle-related genes. The constitutive expression of *c-jun* mRNA in quiescent β TC1 cells is in line with the observation of Piette et al. that in cells transformed chemically or with SV40, the synthesis of AP1, encoded by the *c-jun* gene, becomes independent of growth factors [23]. The expression of *cdc2* at quiescence may be a consequence of SV40 large T antigen expression, probably involved in the immortalization of the initial Beta cells; alternatively, it might be linked to the additional genetic events that have led to the transformation of β TC1 cells. In β TC1 cells, the levels of the *c-fos* and *c-jun* mRNA were cell-cycle dependent. It constituted an early transient response since it was detected within 30 min of serum addition. The early changes in *c-fos* and *c-jun* expression correlate well with the transient character of the induction of these genes by serum in quiescent fibroblasts [21, 24]. The occurrence of *c-fos* together with *c-jun* as an early gene in β TC1 cells suggests that the transcription of AP1-responsive genes triggered by the FOS/AP1 interaction [25–27] constitutes an important step in the progression of these cells towards DNA syn-

thesis. In addition, we have observed that cycloheximide increased the intensity of the *c-fos* signal in quiescent cells, and prolonged its expression in serum-pulsed cells. This superinduction is in agreement with that observed in other cellular systems [28] and has been attributed to the stabilization of short lived *c-fos* mRNA [29]. The constitutive expression of the *cdc2* gene, a homologue of a fission yeast cell division cycle gene, is also of interest since this gene is known to be expressed at a later stage in the cell cycle (end of the G_1 phase), its protein product becoming operational in G_2 [22, 30]. The increase of the *cdc2* signal observed in serum-pulsed cells in the presence of cycloheximide suggests that in β TC1 cells, the expression of the *cdc2* gene might be directly regulated by growth factors. The *c-myc* mRNA was not detectable in quiescent or serum-stimulated cells; in the presence of cycloheximide, only a very weak induction of this mRNA was observed with serum. In contrast, *c-myc* has been shown to be strongly expressed in RIN cells [31]. These results suggest that the regulation of the expression of the *c-myc* gene does not play a pivotal role in the onset of β TC1 cell division cycle.

We have determined the β TC1 cell cycle parameters to be of 21 h, 10 h 30 min and 12 h for the G_1 , S and G_2 /M phases, respectively. The complete β TC1 cell cycle is therefore of 42 to 43 h. Swenne's elegant work using fetal rat islets which have a large proportion of proliferative Beta cells defined for the first time in vitro, the cell cycle kinetics of Beta cells in islets [32]. The cell cycle synchronization was achieved by treatment with hydroxyurea, a drug that collects the cells at the G_1 /S boundary. The length of the G_1 phase was 2.5 h, of S, 6.4 h, of G_2 , 5.5 h and of M, 0.5 h [32]. In the β TC1 cell line, the duration of the S and G_2 /M phases are about twice those determined by Swenne in fetal rat islets. The discrepancy in the timing of events of the β TC1 cell cycle as compared to fetal rat islets essentially resides in the duration of the G_1 phase which is about 8 to 9 times longer in the β TC1 cells than in the fetal islets. There may be an increase in the length of the G_1 phase with age, according to Swenne [33]. Moreover, as recently reviewed by Pardee, the duration of G_1 might be reduced in embryonic cells [1]. While it is not clear that one can extrapolate from fetal rat islet cells to immortalized mouse Beta cells, our results suggest that in view of the cell cycle kinetics, the β TC1 cells behave more like adult cells rather than fetal cells.

Islets in culture constitute invaluable models for identifying substances likely to be physiologically relevant, and this system has permitted the detection of the mitogenic effect of growth factors such as growth hormone [34, 35] and insulin-like growth factor I [36, 37]. Growth factors which are responsible for the resumption of the cell cycle in β TC1 cells have not yet been identified. Preliminary experiments suggest that growth factor effects are not coupled to a cyclic AMP-dependent signal. The results of electroporation of fetal rat islets with several oncogene constructs by Welsh et al. [38] suggested that a mitogenic response occurred when islet cells were transfected with an oncogene (*v-src*) coding for a protein with a tyrosine kinase activity. PDGF is known to induce the state of competence in fibroblasts, i.e. the capacity to

respond to platelet-poor plasma [39] and may have been a good candidate for promoting this response since Swenne et al. showed that PDGF doubled fetal rat islet DNA synthesis [40]. However, Welsh et al. have shown that adult Beta cells require transfection with the gene coding for the B-type PDGF receptor to acquire responsiveness to PDGF [41]. Accordingly, quiescent β TC1 cells were insensitive to PDGF and responded to low concentrations of platelet-poor plasma with the same efficiency as to serum, in contrast with fibroblasts. It therefore seems unlikely that β TC1 cells require PDGF as a competence factor to progress towards DNA synthesis. Our results do not exclude however that other competence factors could be present in platelet-poor plasma. Alternatively the genetic events that have led to the transformation of β TC1 cells may have rendered them independent of PDGF, as described for other transformed cell lines [42]. Moreover, the higher rate of [3 H] thymidine incorporation either with human plasma or serum, as compared to fetal calf serum suggests that other factors or nutrients might be advantageous for β TC1 cell growth. β TC1 cells obtained in the quiescent state and subsequently stimulated by growth factors, provide a good model for studying the short-term effects of purified growth factors, measured by *c-fos* expression, and their long-term effects measured by DNA synthesis and expression of genes appearing later in the cell cycle.

Acknowledgements. The authors are indebted to Dr D. Hanahan for providing the β TC1 cell line. We gratefully acknowledge the Service de cytofluorimétrie analytique et séparative, CNRS, IRSC (Villejuif, France) and particularly Z. Mishal for flow cytometry analysis. We also wish to thank J. Mester and M. E. Forgue-Lafitte for critical reading of the manuscript, D. Lhenry for expert secretarial assistance and Y. Issoulié for prints.

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Received: 5 March 1990
and in revised form: 7 May 1990

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