INTRODUCTION

In malignant tumours, and especially the well-differentiated ones, there is often a predominant cell population whose DNA content is above the normal diploid value. This population constitutes the so-called tumour stem line (Böhm and Sandritter, 1975). In many cases, moreover, there are a number of cells whose ploidy is higher than that of the predominant or modal population. High-ploidy cells are present even in incipient tumours or tumour lines (which are fairly homogeneous in DNA content), and have long been singled out by pathologists, who regard them as a clear sign of malignancy. Today, however, the biological significance of high-ploidy cells is still not known (Liautaud-Roger et al., 1990).

Specifically, one controversial aspect of the behaviour of these cells is their proliferative capacity. While some authors suggest that the proliferative potential of these cells might be reduced (Gustavino et al., 1991) or even nil (Holzner and Golob, 1968; Frankfurt et al., 1985), other authors claim that high-ploidy cells proliferate in a manner similar to that of modal-ploidy cells (Dooley et al., 1991). It is important to learn what their proliferative potential is, since this will help us determine the role of high-ploidy cells in the tumour population. If high-ploidy cells turned out to be incapable of dividing, they would simply represent a marginal tumour subpopulation reflecting an aberrant cell proliferation, and the stem cell function (Pierce, 1978) would be reserved for modal-ploidy cells. If, on the contrary, high-ploidy cells were capable of undergoing productive mitosis, they would be of potential use to the tumour.

This controversy has led us to study cell proliferation in high-ploidy cells. For our research, we selected two murine tumour lines, B16F10 melanoma and 3T3A31M angiosarcoma. According to our results, high-ploidy tumour cells have proliferative capacity, although it is inferior to that of modal-ploidy cells in usual conditions.

MATERIALS AND METHODS

Cells and culture conditions

This study concentrated on two murine tumour lines, B16F10 melanoma and 3T3A31M angiosarcoma. The B16F10 line is a cell variant of B16 melanoma selected for its tendency to generate a high number of pulmonary tumour foci when it is inoculated in the tail vein (Fidler, 1973). The 3T3A31M line was developed from the 3T3A31 line through a process of in vivo and in vitro selection (Zvibel and Raz, 1985). These tumour lines were cultured in DMEM medium to which 10% FCS was added. The cultures were incubated at 37°C in a humidified atmosphere of 10% CO₂ and were used in experiments when they were in a phase of exponential growth. Every two days in the case of B16F10 melanoma, and every three days in the case of 3T3A31M angiosarcoma, the cells were subcultured by enzymic procedures (0.1% trypsin, 2 mM EDTA solution). In order to avoid possible genetic drifts in the line, cells cultured for more than one month were discarded and new ones were drawn from the reserve kept in liquid nitrogen for further experiments.

Measurement of DNA content of cells in different cell cycle phases

The DNA content of the tumour lines was quantified by absorption microspectrophotometry of cells cultured on slides and
stained using the Feulgen procedure. In contrast to flow cytometry, this method makes it possible to visually select the cells to be measured so that cells in interphase, metaphase and telophase can be adequately differentiated. The acid hydrolysis of the Feulgen method was carried out with 1 M HCl at 60˚C for 11 minutes. Later the cells were incubated at room temperature in Schiff reagent for 1 hour. For DNA quantification, a microspectrophotometer equipped with a fast-scanning stage (UMSP-05, Zeiss, Germany) connected to a computer (M-20, Olivetti, Italy) was used. Measuring conditions included a 40× Plan Apo objective (1.0 numerical aperture), 0.8 µm diaphragm and a 40× Plan condenser (0.60 numerical aperture). A 560 nm monochrome light was used for stimulation. For quantification, the integration method was chosen (Caspersson, 1979). Using software developed in our department (Esteban, 1986), we obtained DNA content histograms of the lines. In order to express the DNA content in C units, murine lymphocytes were stained at the same time as the tumour cultures and used as our 2C control.

Three subpopulations at the interphase, metaphase and telophase stages of the cell cycle were selected. The DNA content of at least 600 cells in each subpopulation was quantified. From the DNA-content histogram, we then determined the proportion of cells in the different phases of the cell cycle and the proportion of high-ploidy cells, using the peak reflection method (Dean, 1986).

This method basically consists of plotting the data from the DNA-content histogram onto two Gaussian curves corresponding to the cells in G0/G1 and G2 phases, in the case of the interphase cells. The cells located in the interval between the two curves are the cells in S phase. In the case of cells in metaphase and telophase, the nuclear DNA content is plotted on a single Gaussian curve. In all cases, high-ploidy cells were considered to be those situated to the right of the Gaussian curves.

**Cell proliferation analysed by time-lapse film studies**

The cell cultures were filmed using a system designed in our department that makes it possible to monitor the evolution of the cells over time under programmed conditions of temperature, humidity and CO2 atmosphere. The culture flask was placed in a black perspex box fitted to the stage of an inverted microscope (IM 35, Zeiss). Temperature was maintained at 37˚C by a heating coil connected to a fan that keeps air circulating inside the box. The film was made using a Beaulieu 4008 Z-II camera and super 8 film. The camera was fitted with an optoelectronic sensor causing the film to move forward frame by frame. A computer was used to programme filming conditions, including the frame change rate, which for these experiments was set at one per minute. Black and white photographs were obtained from super 8 photograms using Agfapan-X 100 film.

**RESULTS**

**DNA content and presence of high-ploidy cells in tumour populations in different phases of the cell cycle**

Fig. 1 corresponds to a microphotograph taken of a B16F10 culture stained using the Feulgen method and observed through confocal microscopy. In this figure, next to a group of modal-ploidy cells, a high-ploidy cell can be seen.

The DNA content of the B16F10 cells in the interphase, metaphase and telophase of the cell cycle is shown in Fig. 2A. The DNA-content histogram of the cells in interphase has a classical bimodal pattern corresponding to that of a proliferative cell population. A small percentage of cells located to the right of the Gaussian curve corresponds to the cells in the G2 phase of the cell cycle and is made up of cells whose ploidy is higher than that of the modal population. These are what we have termed high-ploidy cells.

Although most of the metaphases have a DNA content corresponding to that of the modal population following the duplication of their DNA, here the metaphasic population is heteroploid and includes a small percentage of high-ploidy cells (2.4%). Interestingly, the DNA content of the B16F10 telophases is very homogeneous and no high-ploidy cells in telophase were detected in the sample studied.

Fig. 2B shows the DNA-content histograms of 3T3A31M cells in interphase, metaphase and telophase. As with the B16F10 line, here the interphase population includes a percentage of high-ploidy cells, but in this case the percentage is higher (9%). The cells in metaphase also include a subpopulation of high-ploidy cells whose DNA content is practically double that of the modal cells. This subpopulation is approximately 7% of the total. Again, the telophasic daughter cells constitute a very homogeneous subpopulation: in a sample of 600 nuclei, we were unable to find any high-ploidy telophasic cells.

**Evolution of high-ploidy tumour cells recorded in time-lapse film studies**

In analysing the filmed records of the B16F10 and 3T3A31M cultures, and, specifically, in observing the evolution of the high-ploidy cells in metaphase, as well as occasional standard cell divisions giving rise to two daughter cells (which lasted longer than usual), we detected two types of sequence, which are represented schematically in Fig. 3. (a) Mitosis, which had progressed until metaphase, is interrupted, and a single interphasic cell is generated. This phenomenon, called mitotic polypliodization, generates, from a single high-ploidy cell, another cell of higher ploidy (Fig. 3A). This could account for the existence of
high-ploidy metaphasic cells in the absence of the corresponding high-ploidy telophases. (b) High-ploidy metaphasic cells divide productively in a tripolar fashion, giving rise to three daughter cells (Fig. 3B). Careful culture film monitoring of these daughter cells revealed that they reassume an interphasic morphology with evident signs of dynamic cell activity, and provided evidence that the daughter cells from tripolar divisions were, in turn, capable of dividing (Fig. 4). This type of evolution is compatible with the absence or scarcity of high-ploidy telophase cells, since one high-ploidy metaphasic cell can generate three modal-ploidy daughter cells.

**Tripolar mitosis in tumour cultures**

On Feulgen-stained slides, a count was made of the number of tripolar mitoses and their proportion out of the total mitoses in the two tumour lines under study. Fig. 5 shows a tripolar mitosis taken from a B16F10 culture. The results of our quantification of this type of mitosis were as follows: 0.3% tripolar mitoses in the B16F10 line and 2.4% tripolar mitoses in the 3T3A31M line. The higher proportion in the latter line is consistent with the higher incidence of high-ploidy cells in this line.

**DISCUSSION**

Despite the fact that the presence of high-DNA-content tumour cells has long been documented, the role of these cells in the biology of the tumour has not been elucidated. The number of these cells is generally higher in undifferentiated tumours and in advanced neoplastic stages, and their presence is therefore normally associated with a worse clinical prognosis (Auer et al., 1984; Matsuura et al., 1986). However, this is not true of all tumours, since there are cases in which a low DNA content is associated with a poor prognosis (Pui et al., 1991).

One reason why the behaviour of high-DNA-content
tumour cells remains obscure is that most of the current literature on the subject consists of clinical studies aimed at establishing a correlation between the DNA content of a particular tumour and its degree of malignancy. Very little research has been aimed at determining the biological properties of the tumour cells with a high DNA content (Brodsky and Uryvaeva, 1985).

Among the controversial aspects of the biological performance of high-DNA-content tumour cells is their proliferative potential. Cells whose DNA content is above the normal diploid value seem clearly able to proliferate, since the predominant or stem line tumour population frequently has a higher than diploid DNA content (Büchner et al., 1985). What is not clear is the proliferative potential of tumour cells whose DNA content is higher than the modal (that of the stem line), whatever that content may be. Although some authors claim that these cells have a proliferative capacity similar to that of the rest of the population (Dooley et al., 1991), others suggest that the polyploid

Fig. 3. Graphic representation of two types of evolution of the high-ploidy metaphases observed in filmed images of cultures of the tumour lines studied. (A) Shows how a high-ploidy metaphase divides in a tripolar manner, giving rise to three daughter cells. (B) Shows a case of mitotic polyploidization, i.e. the interruption of a mitosis in metaphase to regenerate an interphasic cell of higher ploidy than the original.

Fig. 4. Evolution of a tripolar mitosis in a culture of the 3T3A31M cell line (time-lapse microcinematography sequence). (A) (0 min) Cell that will undergo tripolar mitosis (1) has become rounded and refringent. Next to cell 1 is another, clearly smaller, cell in mitosis. (B) (30 min) Cell 1 has taken on the tripolar shape. (C) (1 h) The three medium-sized daughter cells (2, 3 and 4) have adopted an interphasic morphology. (D) (2 h 30 min) and (E) (4 h 30 min) Cell 2 and the other two daughter cells have moved in opposite directions, thus lengthening the distance between them. (F) (14 h 30 min) Cell 2 has begun mitosis. (G) (16 h 10 min) Daughter cell 4, resulting from the tripolar division of cell 1, begins mitosis. Note the two daughter cells from the division of cell 2 (5 and 6). (H) (18 h 10 min) The third daughter cell (3) derived from the tripolar division begins mitosis. Next to it are the two daughter cells from the division of cell 4 (cells 7 and 8).
cells in general have a longer cell cycle and a smaller probability of undergoing further mitoses (Gustavino et al., 1991), while still others suggest that the mitotic capacity is lost altogether (Holzner and Golob, 1968).

From our results, we deduce that the tumour cells whose ploidy is higher than that of the stem line are capable of dividing, although their cell cycle has certain peculiarities. Thus, in the DNA-content histograms of tumour subpopulations in different phases of the cell cycle, the absence of heterogeneity in the DNA content of the cells in telophase is especially noteworthy, in contrast to the presence of metaphasic cells whose DNA content is above the modal value. This discrepancy suggests that, while, in the tumour lines analysed, the high-DNA-content cells in metaphase are capable of initiating the mitotic process, the division is not completed in the same way as in the rest of the cell population.

The evolution of the high-DNA-content tumour metaphases is a problem whose solution will determine the role attributed to high-ploidy cells (cells whose ploidy is over that of the predominant population) within the context of the heterogeneous tumour population, since this role will depend, among other factors, on their proliferative capacity. Several decades ago, Bader (1959) deduced, on the basis of a much narrower distribution of DNA content in anaphases than in metaphases, that most of the metaphases with DNA content higher than the modal never completed mitosis. This interpretation supported the existence of tumour stem cells with modal ploidy. However, other authors have recently questioned this model, suggesting that all cells complete their division regardless of their chromosome number or their DNA content, and claim that any tumour cell is a potential stem cell (Dooley et al., 1991). These authors find support for this claim in their finding of heterogeneous DNA content in the telophases of certain tumour lines.

Although our results show a discrepancy between the presence of high-ploidy cells over the modal value in the metaphases and the homogeneity of DNA content of the telophases, we do not consider that this entails the inability of high-DNA-content metaphases to complete cellular division, as was suggested previously (Bader, 1959; Pfitzer and Pape, 1973; Frankfurt et al., 1985). It is true that some of these divisions are abortive, as we have verified in filmed records of tumour line cultures, as follows: the cell rounds up and detaches itself from the substratum, initiating a mitosis that is suddenly interrupted, after which the cell reattaches itself to the substratum and resumes an interphase morphology. This phenomenon, known as mitotic polyploidization, has been described in detail by Brodsky and Uryvaeva (1985). Moreover, it is compatible with the ploidy discrepancies between the metaphases and telophases that we have detected.

However, there are other possible evolutions of high-DNA-content metaphases compatible with the absence of corresponding high-DNA-content telophases. For example, chromosome migration to the cell poles could be hindered by the presence of anomalous or especially numerous chromosomes, a situation which is habitual in high-DNA-content metaphases. Indeed, in our filmed records of tumour cultures, we have verified that metaphase lasts longer in hyperploid metaphases, a phenomenon that has also been reported by other authors (Sisken et al., 1985). This increase in the duration of the metaphase may also contribute to the discrepancy in the proportion of high-DNA-content metaphases and telophases.

Moreover, a high-ploidy metaphase is able to evolve through multipolar, especially tripolar, mitosis, as verified in our time-lapse film studies of cell cultures. This phenomenon, which is not exceptional in tumour biopsies (Robbins and Kumar, 1990), is found, in the tumour lines we studied, in proportions ranging between 0.5% and 2.5% of total mitoses. Spontaneous depolyploidization through multipolar mitosis is frequent in other biological models (Afon’kin, 1989), and is compatible with the absence of high-DNA-content telophases in a tumour population with hyperploid metaphases.

In conclusion, our results suggest that high-ploidy tumour cells are capable of proliferating, although this potential is quantitatively inferior to that of the modal cells. The role of these cells in tumour development merits further study.

REFERENCES


(Received 26 May 1992 - Accepted 12 September 1992)