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DNA content analysis of insect cell lines by flow cytometry

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Abstract

The DNA content of insect cell lines (6 lepidoptera, 1 coleoptera and 1 diptera) was determined by flow cytometry. The DNA profiles of the 8 cell lines tested were different. They were characterized by the presence of several peaks (2 to 7) corresponding to different ploidy levels, by differences in the fluorescence intensity of each peak and by the proportion of cells in each peak. Two cell lines (Cf124 and BmN) were constituted of 2 distinct populations of cells. The DNA profiles of the cell lines were stable among the passages and during the length of time culture. This technique was demonstrated to be useful for the detection of mixed cell lines and nucleopolyhedrovirus cell infection, using *Autographa californica* MNPV. The flow cytometry gives interesting results on the cell cycle and the ploidy level; it appears as a good tool for insect cell lines characterization.

Introduction

Continuous insect cell lines are used in various fields of research. They have generally been characterized by morphology and karyotype, then by electrophoretic methods to obtain isoenzyme patterns (Green et al., 1972). However, cell morphology has never been sufficient because changes occur under different conditions and with time in culture. The karyotype study is more reliable except for some cells. Lepidopteran insect cells are one of the exceptions. These cell lines are generally considered as highly polyploid and their nuclei include 200 or more small chromosomes which are very difficult to karyotype (Lynn, 1996). However the images obtained could be artefacts, the number of chomosomes for each cell line remaining constant as observed in the individual living insects (Disney and McCarthy, 1985; Wolf, 1994). Therefore, a homogenous DNA content is expected in the insect cells cultured *in vitro* and some authors have used flow cytometry to characterize the DNA content of insect cell lines (Hilwig and Eipel, 1979a), to study cell cycle kinetics (Fertig et al., 1990) or to determine virus production (Hilwig and Eipel, 1979b; Odier et al., 1993). As it is necessary to find a reproducible method to characterize the DNA content of insect cell lines, in this study, we analyzed the possibilities offered by the flow cytometry to give a constant reproductible DNA profile for each cell line tested.

Materials and methods

Established cell lines

Six established lepidopteran cell lines were used in this study. Two cell lines obtained from embryonic cells of *P. operculella*, ORS-Pop-93 (Po93) (Léry et al., 1995) and ORS-Pop-95 (Po95) (Léry et al., 1997) were cultured in modified Grace's medium (Léry and Fédière, 1990) containing 10% fetal bovine serum

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(FBS). Po93 was maintained at 27°C, and was routinely subcultured every 5 to 7 days when 1 to 2 × 10^6 cells were seeded in each 25 cm^2 flask. Po95 was maintained at 19°C, and was routinely subcultured every 15 to 21 days and seeded at 1 to 2 × 10^6 cells per 25 cm^2 flask. The Po cell lines were used between the 35th and 56th passage. *Choristoneura fumiferana* (Cf124) was a gift from Dr S. Sohi (Sault-Ste-Marie, Canada), *Bombyx mori* (BmN), was a gift from Dr H. Mori (Kyoto, Japan), *Spodoptera littoralis* (Sl) and *Spodoptera frugiperda* clone 9 (Sf9), were gifts from Dr J.M. Quiot and G. Croizier (St-Christol-lès-Alès, France). These 4 cell lines were subcultured for more than 100 passages, and were cultivated under the same conditions as described above for the Po93 cell line. A cell line established from embryonated eggs of *Aedes triseriatus* (At GRIP 1) (Charpentier et al., 1995), named At1, was routinely subcultured for more than 100 passages in Mitsuhashi-Maramorosch medium (Gibco) containing 10% FBS and seeded at 2 × 10^6 cells per 25 cm^2 flask. Finally, a cell line established from larva hemolymph of the Colorado potato beetle *Leptinotarsa decemlineata* (DL1) (Belloncik et al., 1997), named Ld, was routinely subcultured for more than 100 passages in EX-CELL 400 (JRH Bioscience) medium containing 10% FBS and seeded at 2 × 10^6 cells per 25 cm^2 flask.

DNA flow cytometry

Lymphocytes from human peripheral blood (Lc) were prepared using the COULTER Cytometric Oncology System (COULTER DNA-Prep System) and were used as standard for the calibration of the flow cytometer. 100 μl of culture medium containing 2 to 5 × 10^5 cells from the cell lines of each experiment were treated with 2 ml of Krishan buffer (0.1% sodium citrate, 20 μg/ml RNase, 0.3% Nonidet P40 and 50 μg/ml Propidium iodide) for 30 min at room temperature to fragilize the cytoplasmic membrane and to stain the DNA (Rabinovitch and Shankey, 1994). The nuclei were recovered by filtration through 0.22 μm mesh Millipore^{TN} membrane. Nuclei were analyzed with a COULTER EPICS XL-MCL. The protocol used in this study is derived from a protocol commonly used for the flow cytometric assessment of ploidy and proliferation in human studies. Data were collected for 10000 cells from each sample after doublets were eliminated using pulse processing, peak area vs peak height (FS=343 mV; SS=145 mV; FL1=500 mV; FL2=500 mV; FL3=648 mV and FL4=500 mV). For each sample, 3 different counts were made, based on the cell size using forward scattering vs side scattering. When small peaks present in the total cell count could be eliminated using this cell size sorting, they are not taken in account even if visible on the histogram. Average standard errors of the mean (S.E.M.) were based on five counts from different experiments for each sample to give reproductible results.

Experimental mixture of different cell lines

Different cell lines were experimentally mixed after being cultured for 5 days, to evaluate the ability of the flow cytometry to detect and distinguish the cells from different origins. The two cell lines from *P. operculella* (Po93 and Po95) were mixed together as well as Po95 with At1 and Po95 with Sf9.

Influence of different parameters on the DNA profile

Po95 was used to analyze the influence of temperature, number of passages, length of time culture and synchronization treatment on the DNA profile. Cells, cultured during 4 or 7 days at 27°C, maintained at 19°C or 27°C during several passages, treated or not during 24 h with 0.5 mg/ml colchicine for synchronization, were analyzed. The same cells cultured for 36 to 52 passages for the cells maintained at 27°C or 36 to 45 passages for the cells maintained at 19°C, corresponding to a period of 6 months, were also analyzed to test the influence of subculture conditions.

Influence of the viral infection on the DNA profile

A nucleopolyhedrovirus from *Autographa californica* (AcMNPV), a gift from Dr. G. Croizier (St Christol-lès-Alès, France), was used to analyze the influence of the presence of AcMNPV infection on the DNA content of the Sf9 cell line during the early stage of the infection. The AcMNPV inoculum was routinely prepared from Sf9 infected cells supernatant, and filtered on 0.45 μm mesh Millipore^{TN} membrane. Cell cultures, seeded at 4 × 10^6 cells per 25 cm^2 tissue culture flask, were infected after 24 hours with the infectious supernatant AcMNPV multiplied on Sf9. The viral inoculum at a concentration corresponding to 2 virions per cell, was allowed to adsorb for 2 hours, and was then removed and replaced by fresh medium. Samples were taken every 2 hours for 24 hours, then every 24 hours for 72 hours in order to study the kinetics of infection.

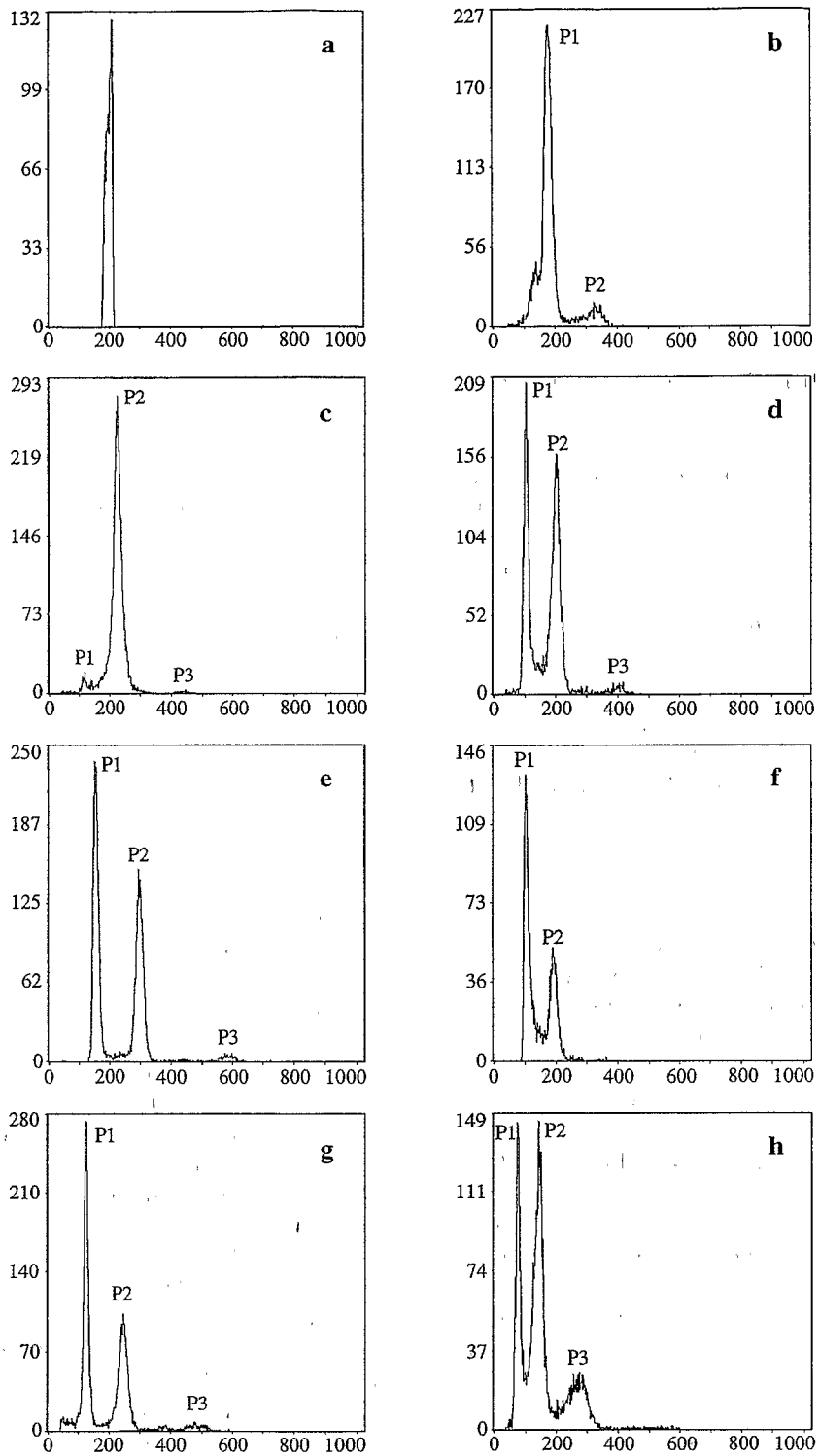

Figure 1. DNA histograms of different insect cell lines by flow cytometry. Units of fluorescence intensity are shown on the *X*-axis. The corresponding number of cells is displayed on the *Y*-axis. (a) human lymphocytes used as internal standard; (b) *Choristoneura fumiferana*, Cf124; (c) *Spodoptera frugiperda* clone 9, Sf9; (d) *Spodoptera littoralis*, Sl; (e) *Phthorimaea operculella*, Po95; (f) *P. operculella*, Po93; (g) *Aedes triseriatus*, At1; (h) *Leptinotarsa decemlineata*, Ld.

Results

DNA profile of insect cell lines

A DNA flow cytometric profile was obtained for each cell line (Fig. 1, Fig. 2). The number of peaks (2 to 4) and the relative DNA content of each peak varied for each cell line. The calibration of the flow cytometer was done with human lymphocytes, which gave a unique peak at a fluorescence intensity (FI) of 200 (Fig. 1a).

On the Table 1, for each cell line, the number of peaks, their relative fluorescence intensity and the proportion of cells in each peak, were reported. Cf124 gave 2 peaks (Fig. 1b), with P1 as the major one. Sf9 gave 3 peaks (Fig. 1c), with P2 as the major one. Sl gave 3 peaks (Fig. 1d), with P1 and P2 as major ones. Po95 gave 3 peaks (Fig. 1e), with P1 and P2 as major ones. Po93 gave 2 peaks (Fig. 1f). At1 gave 3 peaks (Fig. 1g), with P1 and P2 as major ones. Ld gave 3 peaks (Fig. 1h), with P2 as the major one. BmN gave 4 peaks (Fig. 2a), with P3 as the major one. On the Fig. 2b, showing the scattergram of the cell size, 2 populations were selected. Two new profiles could be obtained, corresponding to the 2 populations selected. The first population (20% of the total), corresponding to the largest cells (Fig. 2c), gave 2 peaks: P2 near 185 and P4 near 360. The second population (80% of the total), corresponding to the smallest cells (Fig. 2 d), gave 2 peaks: P1 near 140 and P3 near 280.

Experimental mixture of different cell lines

Po95 cell line was mixed respectively with Po93 (Fig. 3a), At1 (Fig. 3b) and Sf9 (Fig. 3c, d, e, f). With Po93, 4 peaks could be identified, at 95, 160, 190 and 305 FI for cell populations of respectively 32, 40, 14 and 14%. With At1, 4 peaks were seen, at 120, 160, 235 and 300 FI for cell populations of respectively 45, 19, 25, and 11%. With Sf9, the mixture gave 5 peaks at 160, 225, 305, 450 and 610 IF for cell populations of respectively 41, 25, 30, 2 and 2%. As the morphological study of the two cell lines Po95 and Sf9 indicated that there were significant differences between their respective sizes, it was possible, using the morphological cell size discrimination (Fig. 3d) to select the two original cell populations. On the Fig. 3e, a majority of cells (70%) from the Sf9 cell line was selected with 1 major peak at 225 FI. A few number of Po95 were also selected, corresponding to the minor peaks at 160 and 310 FI for cell populations of respectively 8 and 15%. On the Fig. 3f, a majority of cells was selected with the 2 major peaks characteristic from the Po95 cell line, at 160 and 310 FI.

Using the control of Po95 cells alone (Fig. 4a), it was possible to select two different populations in the same sample by the size of the cells (Fig. 4b). The smallest cells represented more than 90% of the total cells and had 2 major peaks: one at 160 FI representing 57% of the cells and one at 305 FI representing 43% of the cells (Fig. 4c). The largest cells gave only 1 major peak at 305 FI representing 64% of the cells and 2 minor peaks at 160 and 595 FI, representing respectively 8 and 18% of the cells (Fig. 4d).

Influence of different parameters on the DNA profile

As shown on the Fig. 5, several factors were tested to determine the stability of the DNA profile of the Po95 cell line. After being subcultured during several passages at 27°C, the Po95 cells showed a general DNA profile (Fig. 5b) similar to the profile of Po95 cells always cultured at 19°C (Fig. 5a). The 2 major peaks were present at the same FIs of 160 and 300 and the proportion of the cells in each P1 was the same, near 52%. The only significant difference was the appearance between P1 and P2 of an intermediate zone corresponding to 16% cells and the decrease of the second peak from 46 to 30%.

The culture time length, 4 (Fig. 5c) or 7 days (Fig. 5e) did not induce any change in the DNA profile of the cell line. Neither the position of the peaks nor the relative proportion of cells for each peak had changed.

No modifications could be noticed in the DNA profile of Po95 cultured during a period of 6 months after being maintained at 19°C (Fig. 1e and 5a) or 27°C (Fig. 5b and 5c). The respective FI positions and the relative cell percentage in each peak were the same.

The synchronization treatment of Po95, maintained at 27°C, with colchicine, induced an inversion in the respective proportions of the major peaks, but the FI position of the peaks did not change. The proportion of cells in P1, which was 60 ± 5% without treatment (Fig. 5c and 5e), became 29 ± 2% when cells were synchronized (Fig. 5d and 5f). In P2, the proportion of cells varied from 25 ± 2% to 46 ± 3%. The intermediate zone between P1 and P2 increased from 14 ± 2% to 21 ± 2%.

Influence of the viral infection on the DNA profile

AcMNPV induced important modifications in the DNA profile of the Sf9 cell line during the first 24

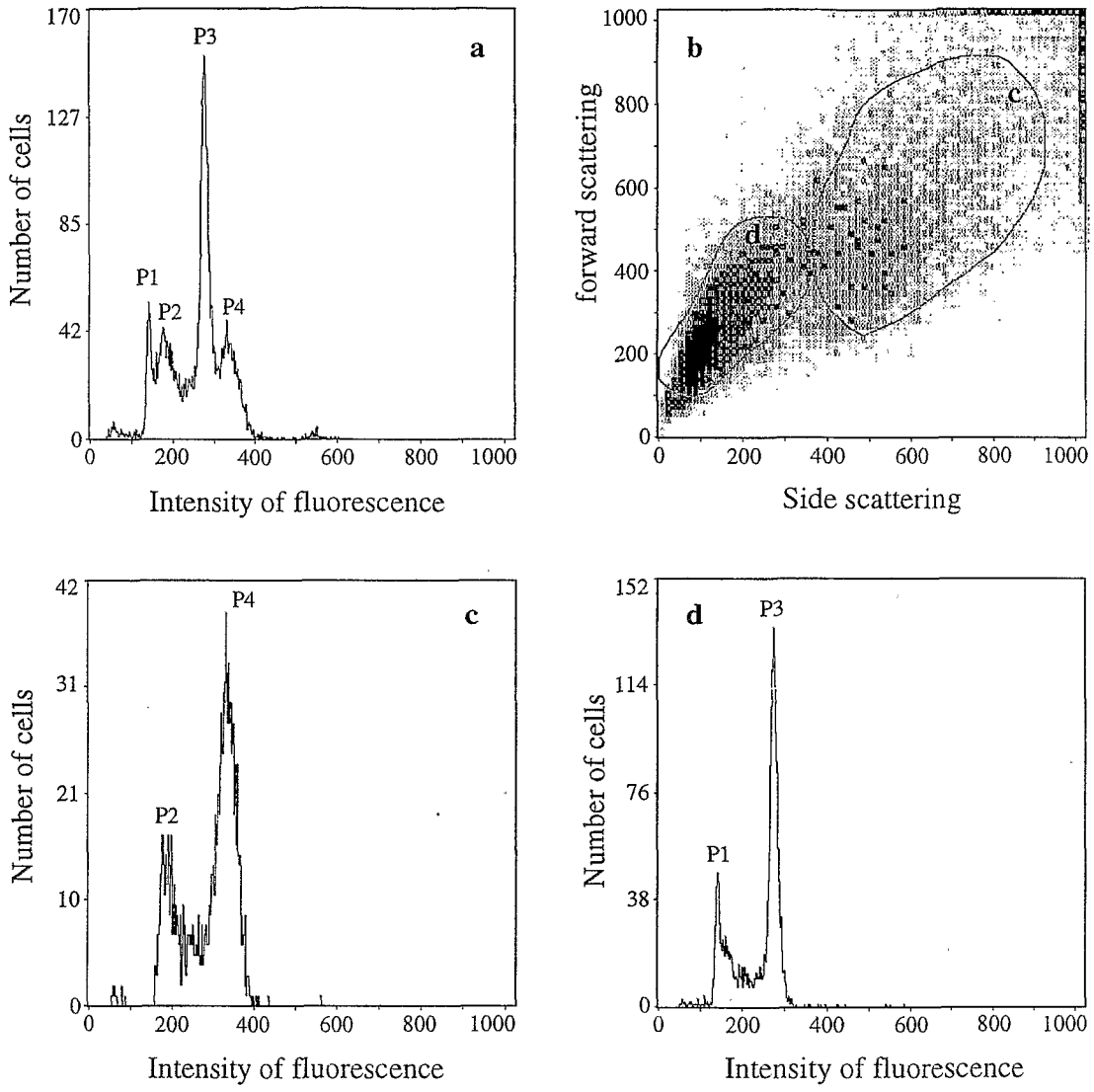

Figure 2. Analysis of the DNA content of the *Bombyx mori* cell line (BmN), by flow cytometry. (a) total cells; (b) scattergram of the cell line size. Each dot represents a measured cell; (c) cell population 1; (d) cell population 2.

Table 1. Comparison between the DNA flow cytometric profiles of several insect cell lines

	Cf124		Sf9		Sl		Po95		Po93		Atl		Ld		Bmn	
Peak	FI	% cells	FI	% cells	FI	% cells	FI	% cells	FI	% cells	FI	% cells	FI	% cells	FI	% cells
P1	175±5	90±9	114±2	5±2	101±2	45±4	157±4	53±5	100±3	66±5	124±4	56±8	74±2	28±4	142±2	12±2
P2	350±5	10±2	225±4	94±3	202±4	52±5	305±6	44±3	197±5	34±4	246±6	40±5	142±3	50±5	185±2	22±2
P3			450±4	1±1	405±5	3±2	600±15	3±2			490±10	4±2	274±5	22±3	280±4	41±4
P4															360±6	24±3

Fluorescence intensity (FI) and percentage of cells (%) are expressed by average + SEM
Cf124: *Choristoneura fumiferana*; Sf9: *Spodoptera frugiperda* clone 9; Sl: *Spodoptera littoralis*; Po95 and Po93: *Phthorimaea operculella*; Atl: *Aedes triseriatus*; Ld: *Leptinotarsa decemlineata*; Bmn: *Bombyx mori*

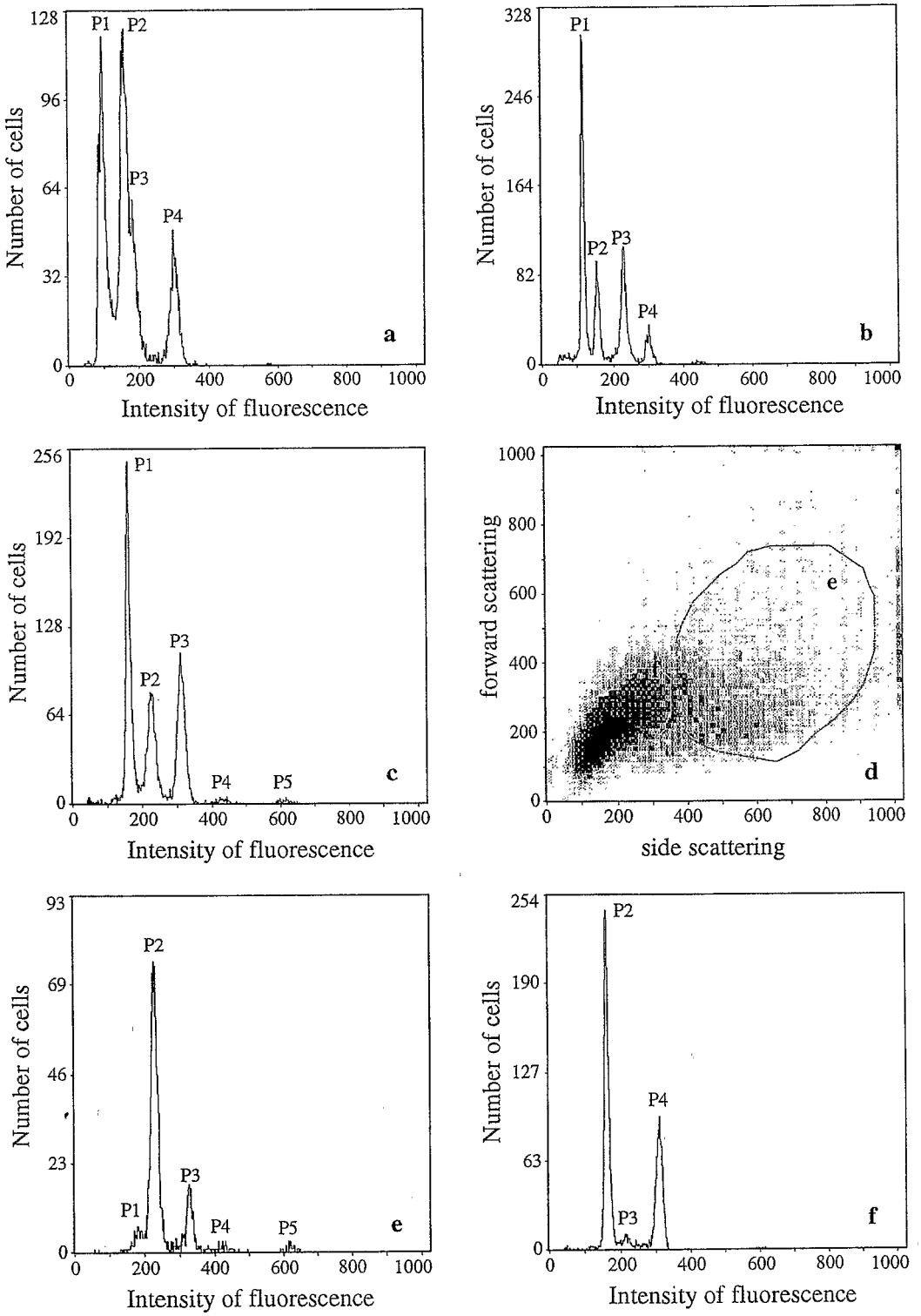

Figure 3. Experimental mixtures of different cell lines analyzed by flow cytometry. (a) Po95 mixed with Po93; (b) Po95 mixed with At1; (c) Po95 mixed with Sf9; (d) scattergram of cell size of Po95 mixed with Sf9; (e) Sf9 cells selected; (f) Po95 cells selected.

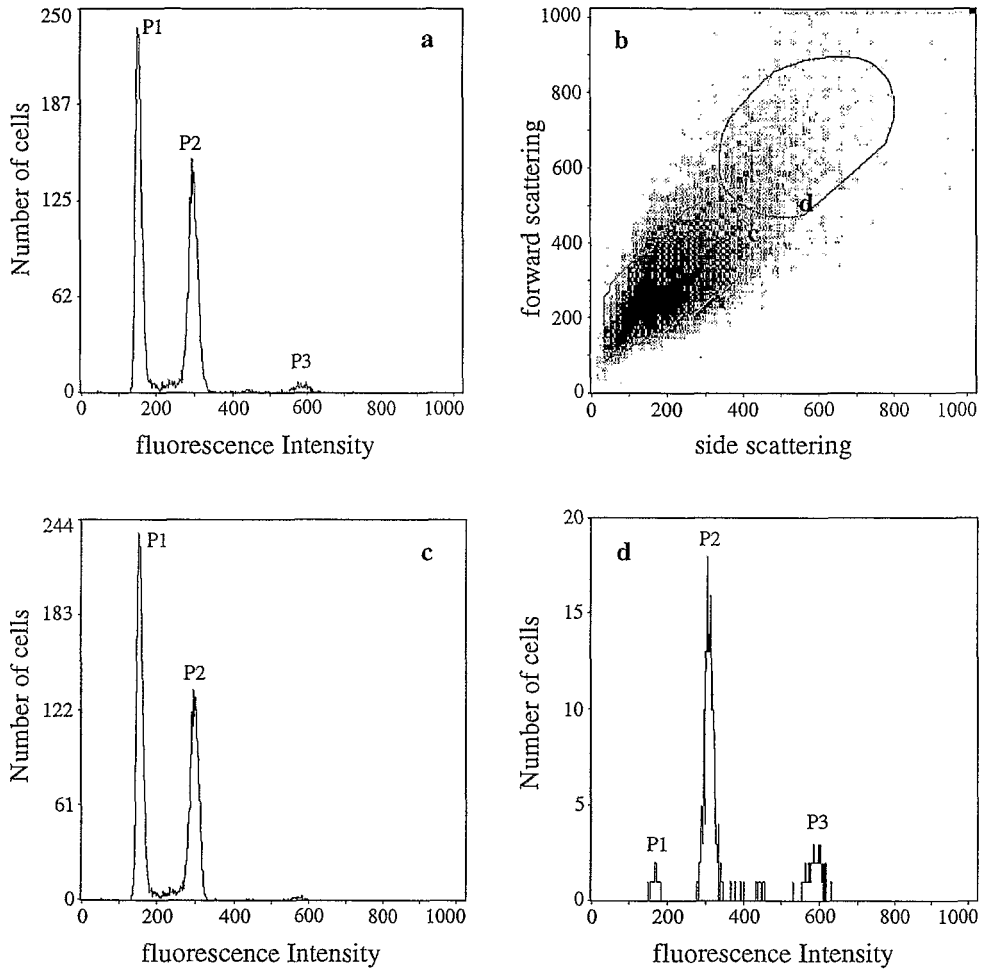

Figure 4. Analysis of the DNA content of the Po95 cell line maintained at 19°C during 45 passages, by flow cytometry. (a) total cells; (b) scattergram of the cell line size; (c) cell population 1; (d) cell population 2.

h (Fig. 6). Four hours after infection, an important peak appeared (Fig. 6b) at 120 FI as the minor peak observed in the control non-infected cells (Fig. 6a). During the next 20 h, the relative importance of this first peak increased (Fig. 6c) until the two peaks were confluent (Fig. 6d). During this first phase, the percentage of cells in the second peak at 225 FI, 43 ± 3%, did not vary significantly. After 24 h, only a single peak with a very large base could be visible (data not shown), without any relation with the distribution of the control cells.

Discussion

The DNA profiles of all the cell lines tested were different. The number of peaks and their respective fluorescence intensity were also different for each cell line. Sf9 was the most homogeneous cell line, as only one major peak corresponding to 94 ± 3% cells was present in the DNA profile. This result was interesting because Sf9 was the only cloned cell line of our study. This result was comparable to the result obtained by Fertig et al. (1990) except that these authors found a more important P1 peak than in our study which may be due to the conditions of culture used in their study (Hilwig and Eipel, 1979a). They also characterized a *Spodoptera littoralis* cell line grown in the same medium used in our experiments. The profile obtained was very similar to the distribution we found, except that only 1 minor peak could be detected in our laboratory. It is possible that aggregates had formed

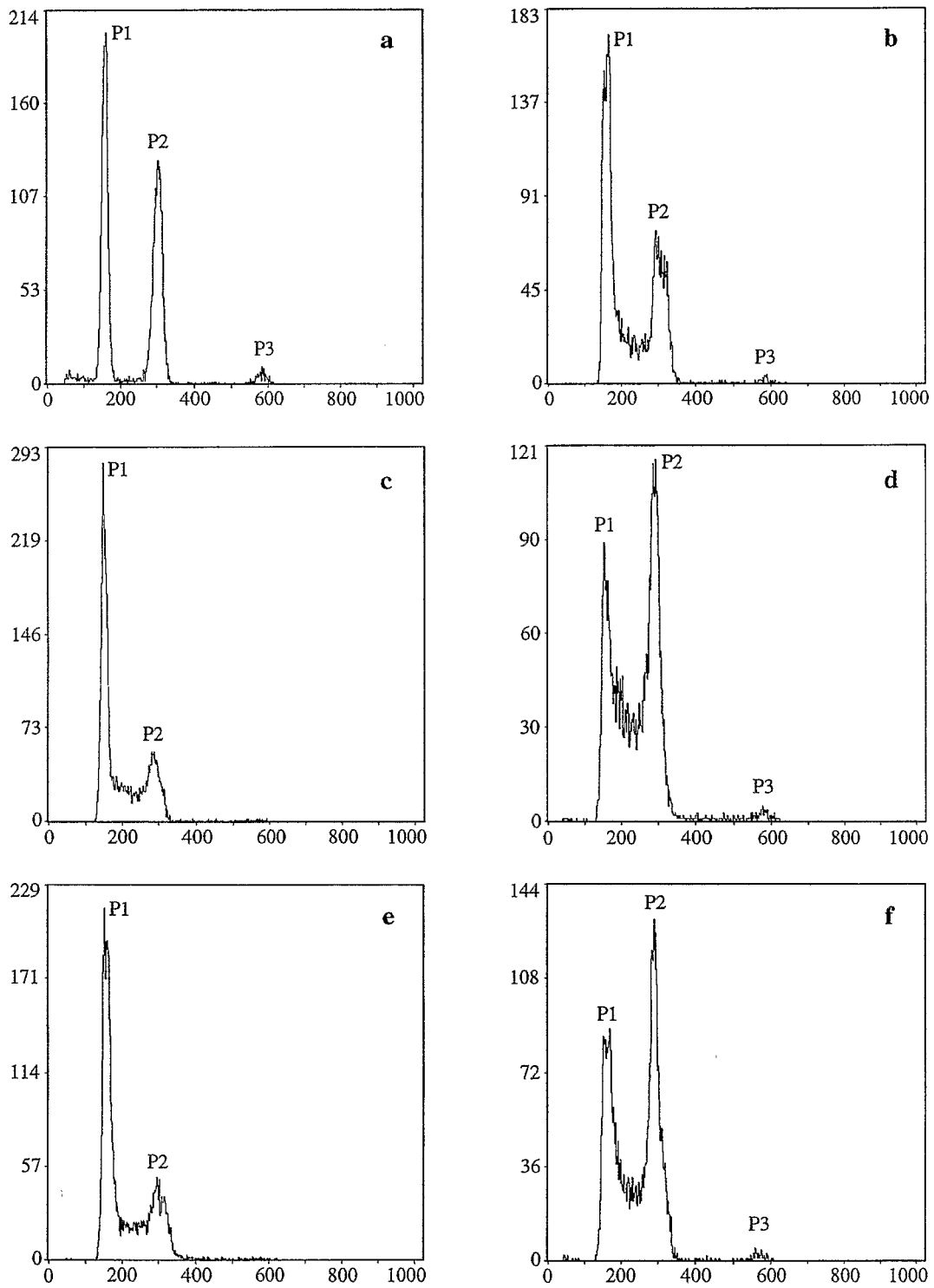

Figure 5. Influence of different parameters on the Po95 DNA content. Units of fluorescence intensity are shown on the *X*-axis. The corresponding number of cells is displayed on the *Y*-axis. (a) Po95 cultured during 36 passages at 19°C after 4 days in culture; (b) Cells cultured during 20 passages at 19°C, then maintained at 27°C during 16 passages, after 4 days in culture; (c) Same cells maintained at 27°C during 16 additional passages after 4 days in culture; (d) Same cells treated 24 h with colchicine; (e) Same cells than in (c), after 7 days in culture; (f) Same cells than in (e), treated 24 h with colchicine.

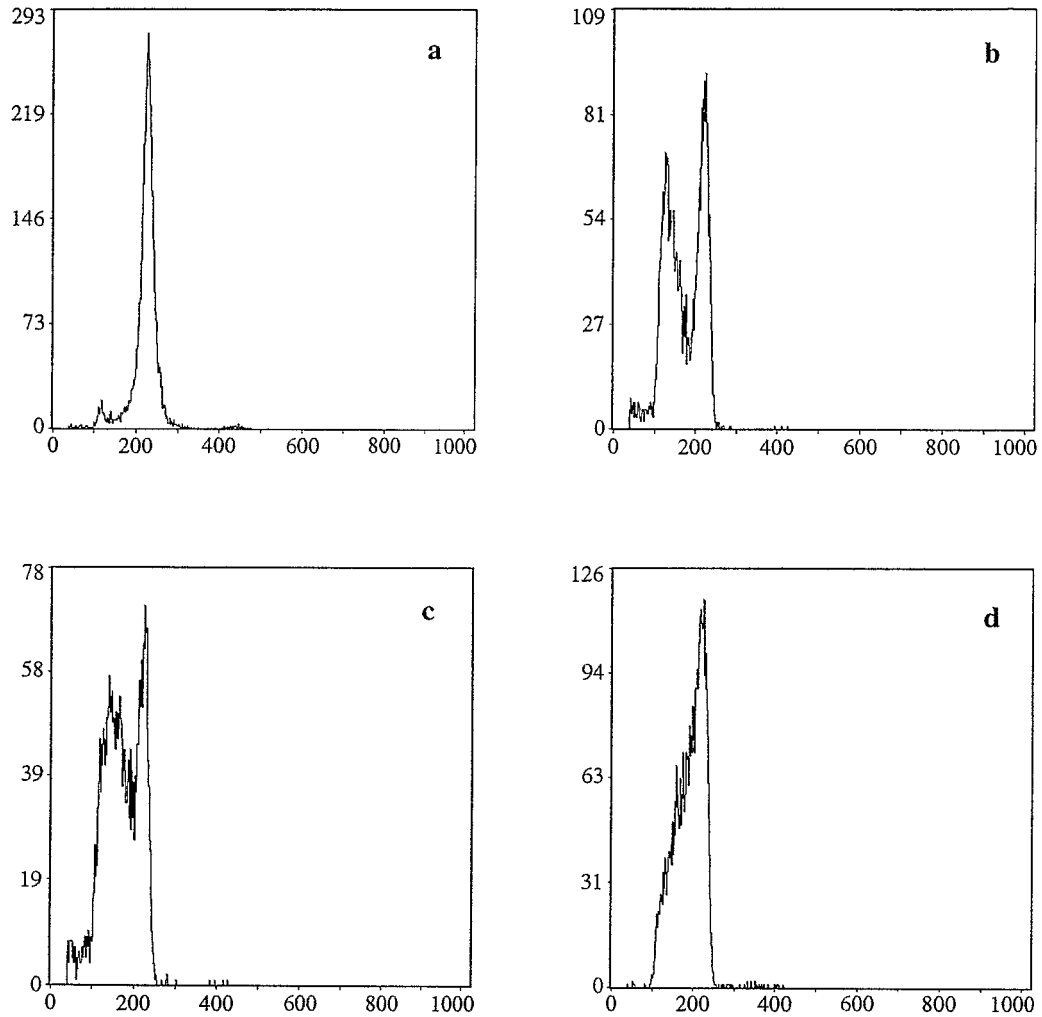

Figure 6. Influence of the AcMNPV infection on the DNA content of Sf9 cell line. Units of fluorescence intensity are shown on the *X*-axis. The corresponding number of cells is displayed on the *Y*-axis. (a) non infected cells at 4 h; (b) infected cells at 4 h; (c) Infected cells at 8 h; (d) Infected cells at 16 h.

resulting in the appearance of small extra peaks. Such aggregates were eliminated from our experiments, and this could explain the smaller amount of minor peaks obtained in our study.

The 2 cell lines established from the same insect *Phthorimaea operculella* at 2 years of interval, resulted in different characteristics in cytometry. Only 2 peaks were detectable in Po93 whereas 3 peaks were present with Po95. Also, the respective FIs of the peaks were different. This difference in DNA content, reflected generally the difference in the size of the cells examined through light microscopy. Because no FI values were mentioned in the 2 previously cited works, no precise comparison between their results and ours could be established, as the major differences between the cells occurred at the FI positions of the peaks. At1 cell line was also found different in the respective FIs, number of cells, and distribution of cell peaks. In all these cell lines, the DNA contents in the individual ploidy levels are related to each other as almost exactly multiple integers. The many peaks in the histogram showed that the cell line contained cells with different ploidy levels (Hilwig and Eipel, 1979a) and there have been several reports of heteroploidy in some Lepidoptera cell lines (Oberlander and Miller, 1987) or diptera (Bello et al., 1997). For all these reasons, it is difficult to determine exactly the cell cycle phases for each ploidy level. For example,

the peak number 2 may consist of G_2-phase cells of the lowest ploidy level (peak 1) mixed with G_1-phase cells of the next higher ploidy level superimposed. But as found when analyzing a homogeneous cell line population, the largest nuclei, corresponding to cells in division, remained at a very low level of 10% or less, among the total cells counted for each experiment. Also, they could be easily eliminated from the results if necessary.

When we examined the profiles obtained with BmN cell line, the different peaks found were not related to each other almost exactely as multiple integers. It was possible, using the cell size discrimination, to select 2 populations of different size, and the peaks, related to each of them. The first population (smallest cells) had 2 peaks at 142 and 280 FI whereas the second population (largest cells) had 2 peaks at 185 and 360 FI. The cell percentage in each peak was the same, with the second peak as the major one. These results clearly showed the high heterogeneity of this cell line and the necessity to clone cell lines.

According to the results obtained previously by karyotype analysis of different insect cell lines (Bello et al., 1997; Ennis and Sohi, 1976; Lynn, 1995), the major peak could represent the cells with $2n$ chromosomes and the minor peaks, the multiploid cells. But Sf9 cell line had its major peak at $4n$ chromosomes as reported previously (Fertig et al., 1990). A specific characteristic of the chromosome organization in the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera, Chrysomelidae) was demonstrated by the DNA profile analysis of Ld cell line. The FI of the 3 peaks were not related to each other as almost exactly multiple integers. The difference of 6 FI could correspond to the unique sexual chromosome, because the karyotype of this species was established to be $2n = 34 + XO$ (male) or $2n = 34 + XX$ (female) (Hsiao and Hsiao, 1982).

When experimental mixtures of different cell lines were done, the respective peaks of each cell line could be obtained. When a morphological study of the two cell lines indicated significant differences between their respective sizes, it was possible, using the cell size discrimination obtained through flow cytometry, to select the two original cell populations. Such a mixture observed in a cell line, could indicate the presence in the cell line of 2 or more cell populations. This result could be very important in the case of natural or chance contamination. As it was always difficult to be sure that a cell line had never been contaminated or mixed in laboratory cell culture conditions, the flow cytometry appears as a good tool, as far as the original profile had been determined previously. The flow cytometry could represent a rapid alternative to other techniques used previously; isoenzyme analysis (Lynn, 1996) or DNA amplification fingerprinting (McIntosh et al., 1996).

The length of the culture did not induce any change in the the cell line DNA profile, which is in agreement with the results obtained previously on *S. litura* (Odier et al., 1993). No modifications could be noticed in the profile of Po95 cultured during a period of 6 months at 19°C or 27°C. Probably, this indicated that our cell lines subcultured for a long period of time, could be considered as stable cell lines. Hilwig and Eipel (1979a) did not give any indication of the number of passages for the two cell lines they have tested (*S. littoralis* and *Mamestra brassicae*), but comparing their results with the DNA profile we have obtained, allows us to think that the number of peaks they had found reflects a great heterogeneity in their cell lines: this could explain the differences obtained with the cell types selected by the culture conditions they used. The cytometry, then, could be a good technique to prove the stability of a cell line in the process of getting established and could certainly give a good image of an established cell line to be used as a reference.

Different profiles could only be found when cells were treated for synchronization. The modifications affected only the proportion of the different cell populations in each peak, but not the FIs of the peaks. As expected, the proportion of the minor peaks is increased whereas the major one decreases. This provides a rapid and reliable method to determine the distribution of the different cell cycle phases and additionally, information on the ploidy state of cell population. The influence of a synchronization treatment with colchicine is in agreement with the presence of spindle apparatus which has been recently shown in lepidopteran cell lines (Traut and Clarke, 1997; Wolf, 1994), even if only partially synchronization was achieved.

Finally, we tested the influence on the Sf9 cell line DNA profile of a viral infection by AcMNPV, as some authors had used this privileged model to screen the production of recombinant proteins by flow cytometry (Eriksson et al., 1996; Farmer et al., 1989). In the first hours of the infection, the DNA content of the peak P2 was not altered. Only the DNA content of the peak P1 was altered. The modifications in the cell DNA content could be induced first by the penetration of the viral DNA (Odier et al., 1993), then

by the beginning of the AcMNPV DNA replication in this cell population. These results could be in relation to observations made by Fertig *at al.* (1990) concerning possible role of stress on the different cell phases. After 24 hours, an overlapping of the fluorescence emission could correspond to the propagation of the viral infection to the whole cell culture. At this stage, the cells contained markedly differing amounts of DNA, which may be due to different numbers of AcMNPV genome per cell (Hilwig and Eipel, 1979a). This point is of great importance in case of abortive viral replication or when the cytopathic effect is low and difficult to detect under light microscope. The flow cytometry could be a convenient method for rapid quantitative monitoring of viral infection to study the permissivity or semi-permissivity of cell lines.

In conclusion, the flow cytometry is a good tool for the study of insect cells *in vitro*. As the DNA profile remained stable during the culture time and among the passages, and because of the differences found between all the insect cell lines tested, the flow cytometry seems to be a convenient method for the insect cell characterization.

Acknowledgments

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