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Time-Lapse Studies on the Effect of Vincristine on HeLa Cells

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Abstract—*Phase-contrast time-lapse studies on the effect of vincristine (VCR) on HeLa cells have been carried out with different VCR doses* (0.0025; 0.005; 0.03 μg/ml) *and different incubation times* (*continuous and* 0.5, 1, 3, 12 hr). *Type and extent of cell damage depend on the applied dose/exposure time relation and on the position of the cells within the cell cycle during VCR treatment. Continuous incubation results in mitotic arrest and subsequent necrosis of all cells entering mitosis, even at VCR concentrations as low as* 0.005 μg/ml. *The fate of short-term treated cells depends on the time of entry into mitosis. All cells entering mitosis during the first* 8 hr *after drug removal are lethally or sublethally damaged; later on regular mitoses also occur. Using cinematography it could be shown directly that VCR-arrested mitoses are no longer capable of further regular proliferation. However, VCR does not only cause lethal cell damage, i.e., necrosis after mitotic arrest, but also sublethal damage leading to pathological divisions after mitotic arrest, with descendants not capable of regular proliferation.*

INTRODUCTION

VINCRISTINE (VCR), a mitotic poison, is widely used in cancer chemotherapy. It leads to mitotic arrest, not only of cells in or shortly prior to mitosis, but also of cells in G_2 and S phase during VCR exposure [1–6]. The question of whether the arrested metaphases are still capable of further proliferation or whether they become necrotic and die is of eminent importance with regard to the use of VCR in tumour therapy. A number of *in vitro* [2, 7, 8] as well as *in vivo* [3, 4, 6] investigations have provided evidence that the arrested mitoses become necrotic and die. Recently, Camplejohn *et al.* [5] were able to show that all VCR-arrested metaphases of the JB-1 ascites tumor become necrotic.

In all of these studies indirect methods are used. Therefore cells can only be followed until the next mitosis and knowledge of only the mean behaviour of a great number of cells can be obtained. In contrast, time-lapse cinematography enables us to study the fate of individual cells and of their descendents before, during and after treatment with cytotoxic agents. Therefore, this method seems particularly apt to investigate in more detail the fate of VCR-arrested mitoses which is still in dispute [9, 10].

The present study with HeLa cells shows that VCR-arrested mitoses are no longer capable of regular proliferation and most of them become necrotic and die after mitotic arrest. A smaller portion is sublethally damaged and undergoes pathological divisions after mitotic arrest, leading to daughter cells not capable of regular proliferation.

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MATERIALS AND METHODS

Culture conditions

HeLa cells were grown in Ham's F 10 medium (Seromed), supplemented with 2.5% fetal calf serum (FCS), 12.5% horse serum (HOS) and antibiotics. The cells were normally grown as monolayer cultures in 75 cm^2 Falcon tissue culture flasks (Falcon Plastics, Los Angeles) and incubated under standard conditions (5–6% CO_2 in air saturated with water vapor, pH 7.2, at 37°C).

For the time-lapse experiments 1×10^5 or 2×10^5 cells were seeded into plastic petri dishes (55 × 15 mm, Falcon Plastics) and incubated for 20–24 hr under standard conditions. Two to three hours before time-lapse photography the bicarbonate buffered me-

dium was replaced by Ham's medium containing 0.102 g $NaHCO_3$ per 1000 ml and buffered with 10 mM Hepes at pH 7.2–7.3. After the normal Petri dish covers had been replaced by tight fitting ones in order to avoid evaporation the Petri dishes were transferred into a controlled temperature (36.5–37.0°C) heated Plexiglas cage which surrounded the microscope.

Cinematographic procedures

Time-lapse photographs were taken with a motor-driven Bolex 16 mm camera (50 mm objective), controlled by an automatic time-lapse system (Paillard-Wild Variotimer Timer MBF-C, Bolex-Wild Variotimer Control Unit MBF-B). The camera was attached to an inverted Wild M 40 microscope, equipped with phase contrast optics, a green filter, a heat protecting filter, long working distance condenser, 10x objective and 6x eyepiece. A specimen-shielding shutter protected the cells from the microscope illumination between exposures. Filming was carried out with 15 mm Gevapan 30 reversal film (Agfa Gevaert) or with Eastman Plus-X negative film (Kodak) with an exposure time of 0.5 sec. Pictures were usually taken with 1 frame/min. Fields containing between 20 and 90 cells were selected for photography.

Vincristine and control experiments

Vincristine sulphate (Lilly) was dissolved in physiological saline. Two series of experiments were carried out.

(1) *Continuous incubations with different VCR concentrations* (0.005 and 0.0025 μg VCR/ml up to about 60 hr).

(2) *Short-term incubations with different VCR concentrations* (0.03 μg VCR/ml for 1 and 3 hr; 0.005 μg VCR/ml for 0.5, 3 and 12 hr). After drug exposure cells were washed 5–6 times in Ringer solution and incubated in fresh medium for up to 100 hr. Filming started about 10–40 min after addition or removal of VCR and in some cases 2–3 hr prior to drug administration.

Untreated HeLa cell cultures with initial

Table 1. Events detected in film viewer

Event	Scoring	Remarks
Rounding of cells	*Beginning*: when the spread interphase cell started contraction in order to assume gradually a refractile circular outline *End*: when the cell had obtained this round shape	Rounding normally occurs when a cell enters mitosis, but it also precedes interphase death
Cell division	*Beginning*: when the round mitotic cell first showed a slight lengthening of shape *End*: when two or more round daughter cells could be recognized	
Cell spreads	At the time of appearance of spread daughter interphase cells	After dividing, the two (or more) daughter cells normally spread rapidly. However, spreading often is delayed in crowded culture dishes or after VCR treatment
Fusion of cells	*Beginning*: when the protoplasm of the fusing cells started confluence *End*: when the cell membrane surrounded the fused cells uniformly without indent	Fusion occurs occasionally between sister cells or closely related cells, but very often between daughter cells during or after multipolar divisions
Agony	*Beginning*: when the luminescent round mitotic cell first showed very small, bright droplet-like protrusions at its outline *End*: when the active phase of the morphologically changing cell structure has stopped, then often giving rise to an oval or nearly round outlined appearance of the cell (could not always be determined exactly)	Before cell death occurs, the arrested mitotic cell passes through a very active phase, during which it appears as a cluster of bright droplets, which changes constantly in morphology. We have denoted this phase as 'agony'. The end of agony is considered as the moment of cell death and the beginning of necrosis
Lysis or disintegration of cells	At the moment of appearance of the empty cell membrane as a dark round shadow	

Table 2. Durations of processes

Process	Duration
Mitotic duration (T_M)	From beginning of rounding of interphase cell until end of cell division
Mitotic arrest	From beginning of mitosis (beginning of rounding) until beginning of agony
Period of time "mitosis until cell death"	Comprises durations of mitotic arrest and of agony
Duration of necrosis	From end of agony until appearance of the empty cell membrane
Period "beginning of mitosis until lysis"	Comprises durations of mitotic arrest, agony and necrosis
Cell cycle duration	From beginning of mitosis of a cell until beginning of the next mitosis of its daughter cell

seedings of 0.5×10^5, 1×10^5 and 2×10^5 cells per petri dish served as controls and were observed up to 137 hr.

Analysis of films

For the quantitative evaluation of the time-lapse films a film viewer with a frame counter (HKS 16 mm film viewer, type LB 1600, West Germany) or an LW international photo optical data analyser with a frame counter (224 A, Mark V, Woodland Hills, California, U.S.A.) were used.

With this method the fate of individual VCR-treated cells and of their descendents, as well as the cell cycle times of untreated and surviving VCR treated HeLa cells, were quantitatively analysed. The times of occurrence of different events (Table 1), for instance, rounding up of the cells or division were determined by recording the number of the frame at which each event was observed. From these data the duration of different processes was deduced (Table 2).

As *regular mitoses* we considered mitoses with the usual duration of about 1 hr and division into 2 daughter cells that continue to proliferate. *Pathological mitoses* show either a considerable prolongation of the mitotic duration (more than 2 hr) or/and multipolar divisions.

Interphase death can clearly be distinguished from death after mitotic arrest, since interphase cells usually contract within 1–3 min, assuming a small irregularly round luminescent shape. Agony starts immediately; its end is sometimes difficult to determine. Necrotic cells arising from interphase cells are usually smaller than necroses after mitotic arrest.

RESULTS

Continuous incubation of cells with 0.005 *or* 0.0025 μg VCR/ml

Mitotic death

(a) 0.005 μg VCR/ml: When HeLa cells are continuously incubated with 0.005 μg VCR/ml medium, all cells entering mitosis during incubation are arrested in mitosis for several hours and subsequently become necrotic. Only mitotic cells that are beyond the late metaphase stage prior to VCR incubation are able to accomplish division. Figure 1 shows the behaviour of HeLa cells; in particular, the passage of two individual cells (marked by arrows) through the beginning of mitosis, mitotic arrest, agony and necrosis.

During the first 2–3 hr of mitotic arrest the cells often form finger-like or bulbous protrusions which are extended and withdrawn within minutes. After several hours of arrest, these mitoses pass through a very active phase of agony during which they appear as clusters of bright droplets constantly changing in morphology. Towards the end of agony the cell membrane appears to tighten again, often giving rise to an oval or round outlined shape of the necrotic cell. Therefore, it is sometimes only possible to distinguish between a mitotic or necrotic cell in a culture dish by considering the history of the individual cell. Several hours after agony the cell membrane of the necrotic cell bursts and the protoplasm extrudes. The empty cell membrane now appears as a dark round shadow. The cell has disintegrated, thus finishing the stage of necrosis.

(b) 0.0025 μg VCR/ml: When HeLa cells are incubated with this lower VCR concentration in the majority of cells the events described above occur. However, some cells (6 out of 47 cells which have rounded up) pass through multipolar division after mitotic arrest. These cells enter mitosis at the earliest 17 hr after VCR exposure and their mitotic duration is prolonged ($\bar{T}_M = 4.0$ hr, $N = 6$).

Interphase death. Whilst in untreated HeLa cells interphase death is rare during the first days after subcultivation, in cultures incubated continuously with VCR for 60 hr about 10% of the cells (0.005 μg/ml: 7 out of 78 cells; 0.0025 μg/ml: 5 out of 47 cells) die during interphase and their deaths occur within 80 hr after subcultivation.

Delayed progression of cells through the cycle. During continuous incubation in VCR the progression of cells—or at least of part of them—through the cell cycle is delayed. However, after a period of 60 hr nearly all of the cells have entered mitosis. The normal cell cycle in VCR-treated surviving cells is about 30 hr (see Table 5).

Short-term incubations with VCR

Incubation with 0.03 μg *VCR*/ml *for* 3 hr *and* 1 hr

(a) *Effect on interphase cells.* Figure 2 shows the effect of a 3 hr incubation with 0.03 μg VCR/ml on the morphology of interphase cells. After 1 hr incubation (Fig. 2a) many and after 2.5 hr incubation (Fig. 2b) all interphase cells show either an irregular outline, due to many small pseudopod-like rapidly moving protoplasmic extrusions, or are bordered by a dark, relatively thick protoplasmic rim. This effect is reversible by removing VCR from the medium (Figs. 2c and d). Seven hours after removal of the drug these interphase cells have regained normal morphological appearance. These alterations cannot be observed with lower VCR doses (Fig. 1).

(b) *Fate of mitotic cells.* (i) 3 hr incubation, 0.03 μg/ml. The scheme in Fig. 3 demonstrates the behaviour and fate of individual cells treated with a 3 hr incubation of 0.03 μg VCR/ml. The individual cells are numbered on the ordinate (1–37) according to their entry into mitosis. For instance, mitosis No. 18 rounds up about 12 hr after removal of VCR and accomplishes a tripolar division after 2 hr of mitotic arrest. One of the daughter cells undergoes interphase death about 6 hr later. The other two daughter cells fuse immediately, even before spreading, and the resulting cell does not proliferate any more until the end of the experiment.

With respect to the fate of the cells, three different time periods can be distinguished:

Firstly, all cells entering mitosis up to about 11 hr after drug removal become necrotic after several hours of mitotic arrest (Fig. 3, mitoses 1–17). Necroses first appear about 14 hr after removal of VCR.

Secondly, most cells rounding up for mitosis from about 12–22 hr either become necrotic (mitoses 19, 22, 23, 24, 27, 31, 32) or pass through pathological divisions after several hours of mitotic arrest. In particular, tri- and tetrapolar divisions occur (mitoses 18, 20, 21, 25, 28, 29, 35, 37) or in a few cases divisions into 2 daughter cells with prolonged mitotic duration occur (mitoses 26, 30). Only few cells undergo regular division (mitoses 33, 34, 36). Daughter cells of the multipolar divisions never show regular proliferation until the end of the 96 hr observation period. Mostly they either die as interphase cells (mitoses 18, 20, 21, 29, 37) or do not proliferate any more (mitoses 18, 21, 25, 28). Only a few of them (mitoses 35, 37) undergo another mitosis, very often a pathological one and after a long period of interphase. Numerous fusions of cells can be observed, either immediately during or after multipolar division or even later. In this experiment at least one fusion has occurred after each multipolar division observed. Fused cells contain usually 2, occasionally 3 nuclei and are seldom capable of passing through another mitosis. This holds true also for bi- or multinucleated cells occasionally found in untreated HeLa cell cultures. Pathological divisions mostly are characterized by aberrant cytokinesis.

Thirdly, all cells entering mitosis later than 22 hr after drug removal accomplish regular mitosis (Fig. 3, upper row). The descendents of quite a number of these mitoses could be followed through two successive cell cycles. There is a period of about 10 hr with complete lack of mitoses between the first and second group of mitoses and a shorter one between the second and third group of mitoses.

Repetition of the experiment rendered principally the same results, except that the timing of the periods was slightly different and that pathological mitoses occurred preferentially as divisions into two daughter cells with prolonged mitotic duration ($\bar{T}_M = 3.9$ hr).

The percentages of necroses, pathological divisions and regular mitoses are listed in

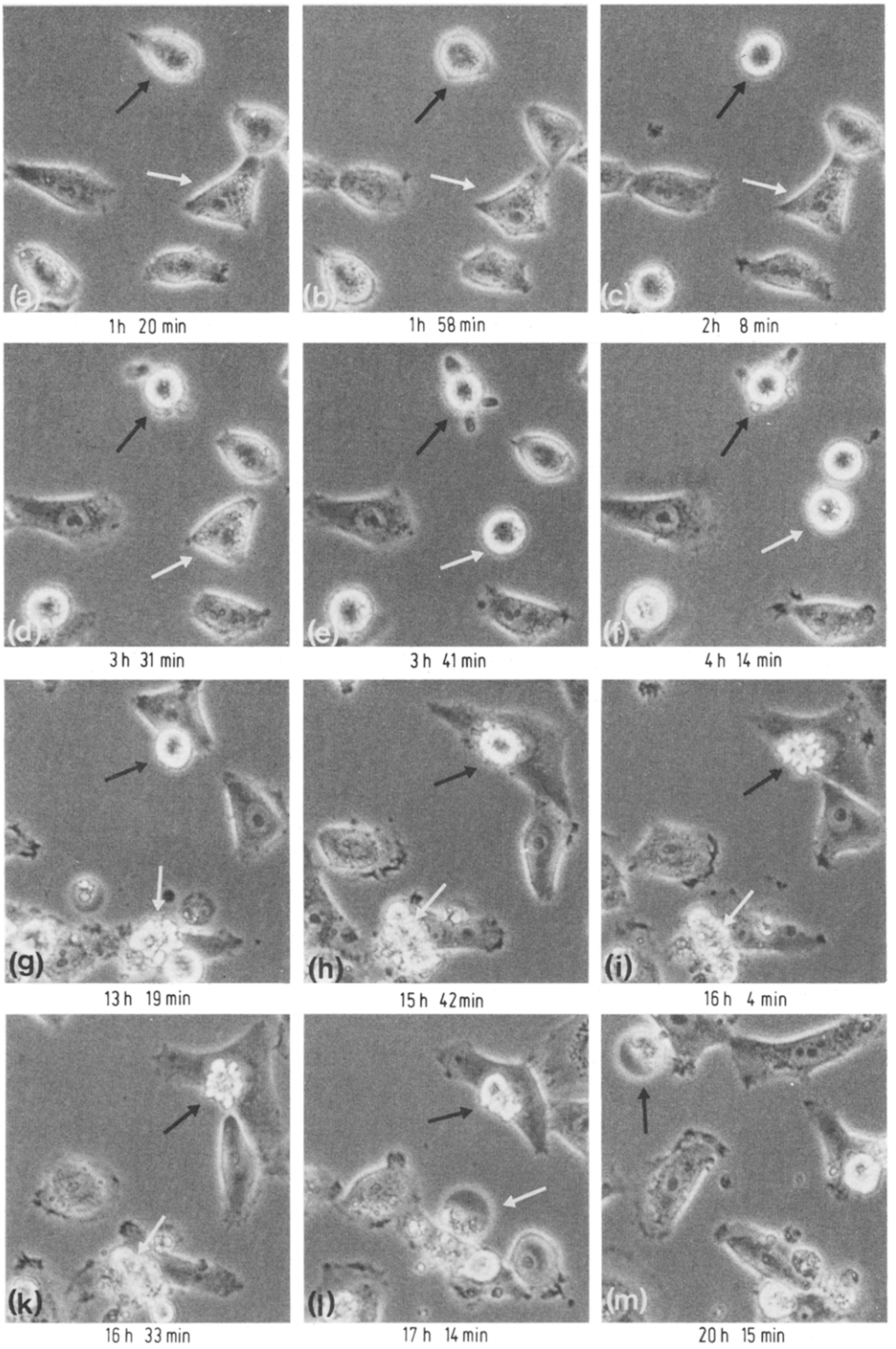

Fig. 1. *HeLa cells during continuous incubation with* 0.005 μg *VCR*/ml. *All cells entering mitosis during continuous incubation become arrested in mitosis for several hours and subsequently undergo agony, necrosis and lysis. Two individual cells can be followed from beginning of mitosis until lysis. The given times denote the time after addition of VCR. Cell* 1*: Rounding up* (*b, c*), *mitotic arrest* (*d–g*), *protrusions during mitotic arrest* (*d–f*), *agony* (*h–l*), *empty cell membrane after lysis* (*m*). *Cell* 2*: Rounding up* (*d, e*), *mitotic arrest* (*f*), *agony* (*g*), *necrosis* (*h–k*), *empty cell membrane after lysis* (*l*).

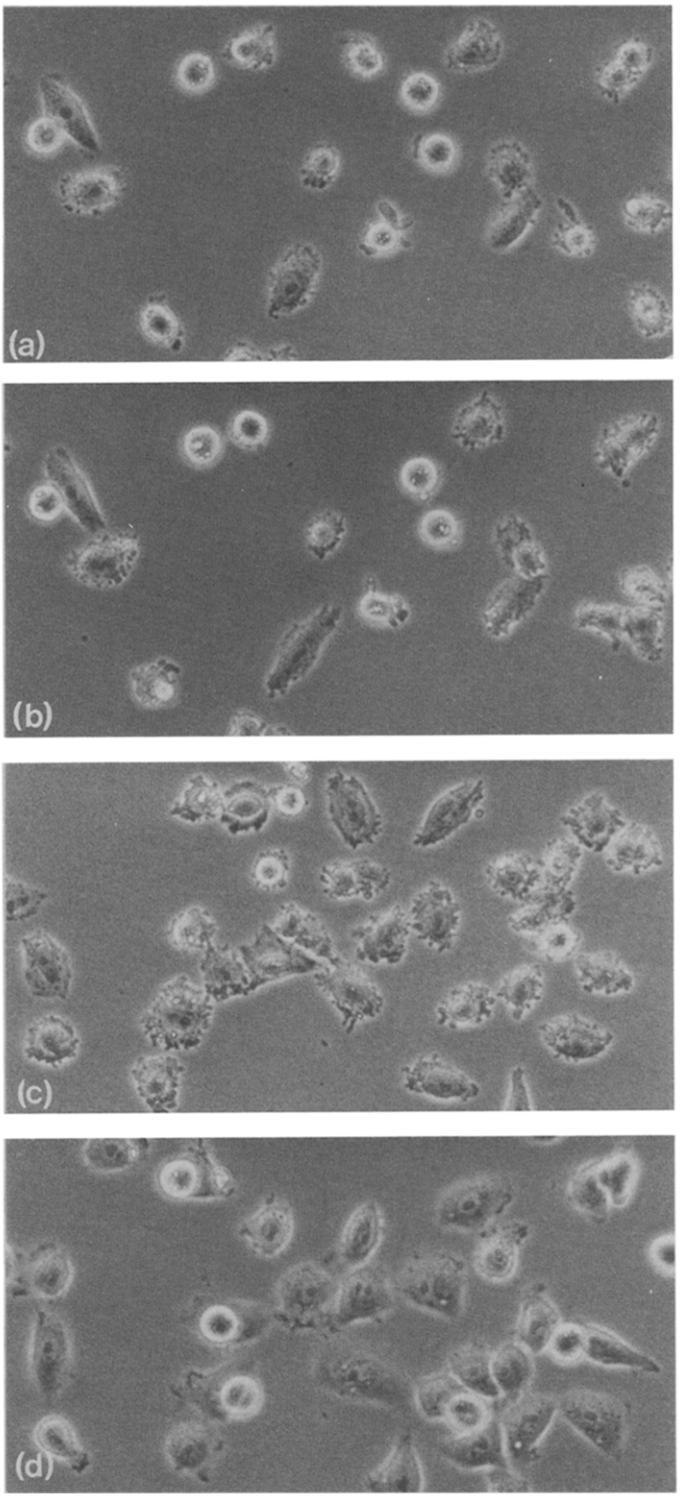

Fig. 2. Influence of 0.03 µg *VCR*/ml *on the morphology of interphase cells. A concentration of* 0.03 µg *VCR*/ml *causes severe morphological changes of interphase cells* (*a, b*), *which are reversible after removal of the drug* (*c, d*). *a and b:* 1 hr *and* 2 1/2 hr, *respectively, after addition of VCR; c and d:* 30 min *and* 7 hr, *respectively, after removal of VCR.*

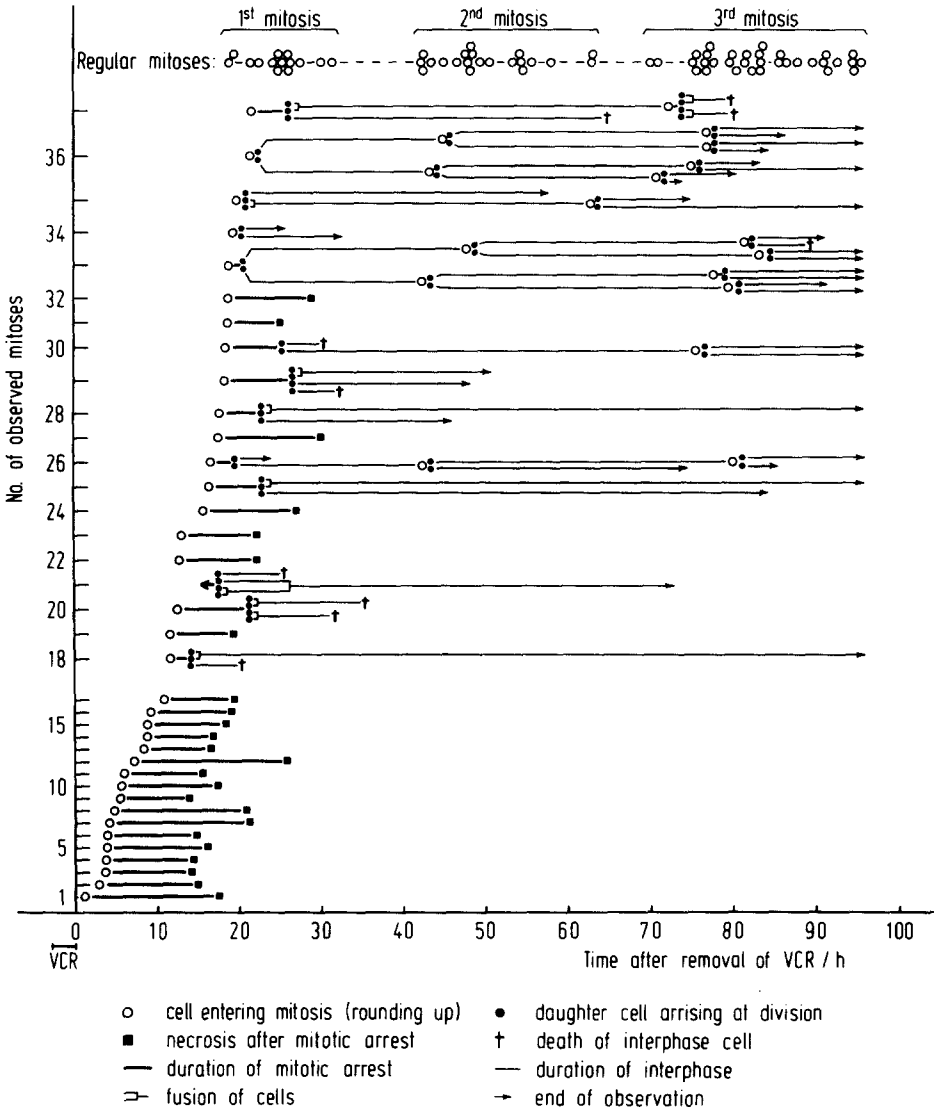

Fig. 3. Fate of HeLa cells after 3 hr *incubation with* 0.03 μg *VCR*/ml. *HeLa cells entering mitosis after removal of VCR can undergo different fates — necrosis, pathological division or regular division — depending on the time of entry into mitosis.*

Table 3, together with the results of other short-term incubations.

(ii) 1 hr incubation, 0.03 μg/ml. When HeLa cells are incubated with the same dose for only 1 hr, the effect of VCR is reduced (Table 3). Compared to a 3 hr incubation, more cells divide regularly and there are more pathological mitoses and less necroses. The time periods of mitoses with the fate of necrosis, of pathological division and of regular mitosis are overlapping. Regular mitoses do not occur earlier than 10 hr after drug removal.

Incubation with 0.005 μg *VCR*/ml *for* 0.5, 3 *and* 12 hr. When HeLa cells are short-term incubated with lower doses of VCR, increased incubation times are necessary to obtain the same effect with respect to mitotic arrest and damage of the cells.

Incubation with 0.005 μg VCR/ml shows only little effect after exposure for 0.5 and 3 hr. After a 12 hr incubation the extent of cell damage is comparable to that of a 3 hr incubation with 0.03 μg VCR/ml (Fig. 3, Table 3). However, the group of pathological mitoses is enlarged and appears soon after drug removal. Regular mitoses appear at the earliest about 9 hr after drug removal. In accordance with the 3 hr incubation with 0.03 μg VCR/ml, there is a time interval of about 9 hr with no cells entering mitosis, following the first regular mitoses.

Table 3. Fate of HeLa cells short-term incubated with VCR

Kind of treatment			Fate of cells in %						
				Pathological divisions					
Concentration of VCR (μg/ml)	Incubation time (hr)	Number of cells present after drug removal	Necroses after mitotic arrest (%)	Multipolar divisions (%)	$T_M > 120$ min (%)	Regular mitoses (%)	Interphase death (%)	No. divisions (%)	Cells with fate not discernable (%)
0.03*	3	97	46.4	7.2	10.3 (17.5)	21.6	2.1	0	12.4
0.03	1	74	28.4	9.5	13.5 (23.0)	41.9	0	2.7	4.1
0.005†	12	74	33.8	21.6	2.7 (24.3)	16.2	0	0	25.7
		[55]	[45.5]		[32.7]	[21.8]	0	0	—

*Results of two experiments.

†The percentages refer to all cells present after removal of the drug. The percentages added in brackets refer only to those cells that are not lost from the field of view during observation.

Duration of mitotic arrest, agony and necrosis and of cell cycle times

Mitotic arrest, agony and necrosis. Table 4 contains the durations of mitotic arrest, agony and necrosis for continuous incubation as well as for short-term incubation with different VCR doses. On the average, mitotic arrest lasts about 7–9 hr, agony about 1 hr, necrosis 3–5 hr and the period "beginning of mitosis until lysis" 11–15 hr. The durations of these phases are independent of the kind of treatment.

Cell cycle times. Cell cycle times determined after short-term incubations with VCR and in control experiments are given in Table 5, together with the corresponding ranges.

After *short-term incubation* with different doses and incubation times the descendants of regular mitoses were followed through one or two successive cell cycles. The individual cycle times vary considerably. On the average they are shorter for the first than for the second cycle.

In *control experiments without VCR* the mean cycle times of HeLa cells are considerably longer (~40 hr) than after short-term incubation with VCR (25–30 hr) and show a high

Table 4. Durations of mitotic arrest, agony and necrosis of HeLa cells treated with VCR

VCR dose (μg/ml)	Kind of treatment	Mitotic arrest Mean ± S.D. (hr)	Agony Mean ± S.D. (hr)	Necrosis Mean ± S.D. (hr)	Beginning of mitosis until lysis Mean ± S.D. (hr)
0.0025	Continuous incubation	7.0 ± 4.8 (N=34)	1.06 ± 0.33 (N=34)	3.9 ± 2.2 (N=30)	11.0 ± 2.7 (N=30)
0.005	Continuous incubation	8.2 ± 2.8 (N=64)	0.90 ± 0.32 (N=65)	3.4 ± 1.5 (N=63)	12.2 ± 2.6 (N=60)
0.005	12 hr incubation	7.9 ± 3.1 (N=30)	1.21 ± 0.39 (N=27)	3.3 ± 1.7* (N=17)	12.8 ± 4.1* (N=16)
0.03	3 hr incubation	9.3 ± 3.3 (N=46)	1.27 ± 0.49 (N=43)	5.6 ± 3.4 (N=35)	15.6 ± 5.0 (N=36)
0.03	1 hr incubation	6.9 ± 6.0 (N=18)	1.35 ± 0.62 (N=16)	4.6 ± 2.5 (N=14)	13.7 ± 7.1 (N=14)

*These values might in fact be slightly higher, as a number of the evaluated cells had already begun mitosis before observation started, or had not yet lysed at the end of observation.

Table 5. Cell cycle durations of HeLa cells after short-term incubation with VCR and of untreated controls

Kind of treatment					Duration of:		
VCR dose (μg/ml)	Incubation time	Number of seeded cells	Regularly dividing cells (%)	Duration of observation (hr)	First cycle mean ± S.D. (hr) [range (hr)]	Second cycle mean ± S.D. (hr) [range (hr)]	Third cycle mean ± S.D. (hr) [range (hr)]
0.005	3 hr	100,000	ca. 80	62	26.0 ± 3.5 [20.8 – 31.4] (N = 19)	—	—
0.005	12 hr	170,000	ca. 20–25	110	30.6 ± 7.7 [20.1 – 45.7]* (N = 10)	32.5 ± 5.4 [22.5 – 41.0] (N = 11)	—
0.03	3 hr	200,000	ca. 20–25	96	25.6 ± 3.0 [22.0 – 31.6] (N = 13)	34.0 ± 4.1 [26.7 – 42.2] (N = 20)	—
Control		50,000	ca. 85–90	137	40.4 ± 6.7 [28.2 – 57.4] (N = 17)	39.6 ± 5.2 [29.0 – 47.6] (N = 17)	43.5 ± 5.6 [32.0 – 48.1] (N = 7)
Control		100,000	ca. 90–95	91	40.3 ± 4.5 [33.9 – 48.7] (N = 18)	43.0 ± 5.5 [31.4 – 50.5] (N = 11)	—
Control		200.000	ca. 80–85	96	45.7 ± 7.6 [36.6 – 57.3] (N = 14)	No second cycle up to 96 hr	—

*Only one value above 37 hr.

degree of variability. There seems to be an increase in cycle time as soon as a certain cell density is reached (i.e. experiment starting with 2×10^5 seeded cells).

DISCUSSION

Fate of arrested mitoses

Necroses. The present time-lapse studies show that the VCR-arrested mitoses are no longer capable of regular proliferation. Most of the arrested mitotic cells become necrotic and die after several hours of arrest. This is in good agreement with the results of George *et al.* [7] who found the metaphase arrest of HeLa cells by VCR not to be reversible. In 1964 Frei III *et al.* [11] concluded from *in vivo* experiments that most of the VCR-arrested metaphases do not survive. Similar results were found by Jellinghaus *et al.* [3] for jejunal crypt cells of the mouse. Recently Camplejohn *et al.* [5] showed that all VCR-arrested metaphases of the JB-1 ascites tumour become necrotic.

The mean durations of mitotic arrest, agony, necrosis and "beginning of mitosis until lysis" of VCR-treated HeLa cells are quite similar regardless of the dose and time of incubation with VCR (Table 4). The cumulative duration of mitotic arrest, agony and necrosis of about 11–15 hr agrees well with the finding of Madoc-Jones and Mauro [2] who observed that HeLa cells treated with VCR or vinblastine (VLB) begin to lyse after about 12 hr metaphase arrest. However, there seems to be considerable variation between different cell types. For instance, metaphase arrest of the JB-1 ascites tumour cells lasts about 4 hr and necrosis about 17 hr [5], while necrotic murine jejunal crypt cells were already found 1 hr after the occurrence of the first VCR-arrested metaphases [3].

The deficit of descendants of the mitotic necroses after short-term incubation with VCR leads to a 'negative synchronization', i.e., periods of complete lack of mitoses between the groups of successive regular mitoses.

Pathological divisions. VCR does not only lead to direct lethal damage of HeLa cells resulting in necrosis and lysis of the arrested mitoses, but also causes sublethal damage leading to pathological divisions after mitotic arrest. The lethal damage of these cells becomes manifest much later, when the daughter cells prove to be not capable of regular proliferation. Thus, the portion of VCR-damaged cells is much greater than the percentage of lethally injured cells observed soon after VCR treatment (Table 3). In contrast to Klein [10], regular proliferation of multinucleated cells has not been observed in the present experiments.

Multipolar divisions as seen under the influence of VCR also occur rarely in untreated HeLa cells. Their increased appearance, however, is not specific for VCR but seems to be a more general expression of cell damage. An increase in multipolar division has also been observed after irradiation with low X-ray doses [12, 13], after treatment with other agents such as colcemid [14], or BCNU [1,3 bis(2-chloroethyl)-1-nitrosourea] [15] and in cell cultures under poor cultural conditions (personal observation). Krishan [16] found the reason for multipolar mitoses after vinblastine treatment to result from continued centriolar duplication during the mitotic block.

Damage of interphase cells

Morphological changes. The morphological changes of interphase cells incubated with 0.03 μg VCR per ml correspond to the "ruffling of the surface" that has been observed by George *et al.* [7] in HeLa cells during incubation with 1 μg VCR/ml. Alterations of the cell shape, however, are not specific for VCR, since they have also been found in cultures exposed to other agents such as VLB ([17], own observations) or actinomycin D and puromycin [7].

Interphase death. The occurrence of interphase death is not very frequent in VCR-treated HeLa cells except for descendants of pathological mitoses. Hurtwitz and Tolmach [18] also discerned lysis from interphase cells and from mitotic cells after X-irradiation. Interphase death of VCR-treated cells suggests that the cytocidal action of VCR might not only be due to the stathmokinetic effect but also to membrane alterations as proposed by Krishan and Frei III [17] or to the inhibition of the synthesis of macromolecules [19].

Effect of VCR dose, exposure time and position of cells within the cell cycle

'Dose–exposure time' relation. The type and extent of damage of HeLa cells by VCR essentially depend on the dose applied and the duration of exposure. Incubation with 0.03 μg VCR/ml for 3 hr results in a very high percentage of mitotic necroses and considerably less pathological divisions, while reduction of exposure time or VCR dose leads to a shift from lethally to sublethally injured cells (Table 3). In order to obtain the same extent of damage increasing exposure times are required, if the VCR dose is reduced. Both a 12 hr exposure to 0.005 μg/ml and a 3 hr incubation with 0.03 μg/ml result in about 70% damaged cells. These findings agree well with the investigations of Jackson and Bender [20] of the effect of different doses and exposure times on the extent of cell damage of L 1210 and CEM cells.

Effect of VCR on cells in different cycle phases. Based on the cycle phase durations of HeLa cells, the cell age at the time of VCR exposure can be determined. Those cells that enter mitosis first (up to 8 hr) after removal of VCR and are lethally or sublethally damaged must have been in G_2 or S phase, or at least late S phase, during VCR incubation. The cells which enter mitosis during the intermediate time period and undergo different fates might have been in middle or early S or in late G_1 phase. The group of regularly dividing cells can be assumed to have been in G_1 phase since these cells do not enter mitosis before 10 hr after VCR removal.

A more exact determination of the cell age during VCR treatment is difficult, particularly for the intermediate group, since the individual cycle times vary considerably and the mean cycle times of untreated and VCR-treated cells differ markedly (see below). However, it can be stated that cells that were in G_2 and S, or at least late S phase, during VCR treatment, are lethally or sublethally damaged, while cells in G_1, possibly except late G_1, are unaffected by VCR.

The VCR-sensitive cycle phases of HeLa cells derived from the present experiments agree well with studies of Madoc-Jones and Mauro [1] on HeLa and Chinese hamster cells and with *in vitro* and *in vivo* studies of Jellinghaus *et al.* [3, 4] and of Ernst [6]. According to these authors, VCR affects cells in S and G_2 in such a manner that they are arrested during the next mitosis. Similarly, Camplejohn *et al.* [5] found metaphase arrest and subsequent death of all JB-1 ascites tumour cells that were in G_2 and the last two-thirds of S phase at the time of VCR injection.

Cell cycle of VCR-treated and untreated HeLa cells

The mean cycle time of HeLa cells after short-term incubation with VCR (Table 5) is within the range of 25–30 hr. This time is the same as that determined by other authors for untreated HeLa cells [21]. However, the cell cycle of untreated HeLa cells in the present experiments is prolonged. This seems to be due mainly to the use of Petri dishes during the filming procedure. Using culture flasks we

observe cycle times for untreated HeLa cells that are in accordance with the literature. Furthermore, a shift towards lower pH values was observed in untreated HeLa cell cultures, while the pH remained essentially constant after short-term incubation with VCR. This acidification of the medium might be the reason for the prolonged cycle time of untreated HeLa cells. Verhaegen and Rousseau [22] also observed an increasing inhibition of cell proliferation with increasing acidification of the culture medium by HTC cells.

The high degree of variability of individual cycle times, even for sister cells, both in untreated and short-term incubated HeLa cell cultures (Table 5) corresponds to findings on hepatoma cells [23] and on HeLa cells, in particular after X-irradiation [13, 24] and on rat 9L brain tumour cells prior to and after BCNU treatment [15]. In agreement with earlier findings of Madoc-Jones and Mauro [1] the progression of cells through the cycle during continuous incubation with VCR is delayed.

Mechanism of action of vincristine

There is a lot of evidence that VCR, as well as VLB, cause their cytolytic effects mainly by interaction with tubulin, the soluble protein component of microtubules [25–27]. This results in a prevention of spindle tubule formation and a disturbance of the development of a functioning spindle apparatus. According to Owellen *et al.* [25, 26] the primary mode of action of the Vinca alkaloids is the binding to tubulin and tubulin-like proteins. VCR and VLB also block polymerization of soluble tubulin into microtubules in a dose dependent manner [27]. Electron microscopic studies of HeLa cells revealed the absence of spindle tubules in VCR-arrested cells as well as the inhibition of migration of the centrioles to opposite poles of the spindle [7]. Malawista *et al.* [28] even observed spindle dissolution during exposure to very high VCR concentrations and incomplete recovery after removal of the drug, "with the frequent induction of small tri- and tetrapolar spindles". Robbins *et al.* [29] found pericentriolar changes which are probably related to spindle tubule formation, occurring from beginning of DNA synthesis until late prophase.

The present observations on the fate of VCR-damaged cells and on the sensitivity of cell cycle phases to VCR are in good accordance with the mechanism of action of VCR described above.

Vincristine in the so-called "synchronization therapy"

VCR, widely applied in cancer chemotherapy, has been used by Klein and coworkers [6, 15, 16] in a so-called "synchronization therapy". A concentration of 0.03 μg/ml, as applied in animal experiments and in the therapy of human tumours, is assumed by these authors not to be cytotoxic and to result in partial synchronization of the tumour cells. The present time-lapse studies with HeLa cells directly demonstrate that the VCR-arrested mitotic cells are no longer capable of further regular proliferation. In accordance with Jellinghaus *et al.* [3, 33, 34] and Camplejohn *et al.* [5], we therefore believe that VCR cannot be used for cell synchronization.

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