Europ. J. Cancer Vol. 15, pp. 17–29
© Pergamon Press Ltd. 1979. Printed in Great Britain

0014-2964/79/0101-0017/$02.00/0

Cytokinetic Effects of Continuous Infusion of Vincristine in Head and Neck Carcinoma*†

GIANNA BADARACCO,‡ MARIELLA MORELLI,§ CARLO NERVI|| and GIUSEPPE STARACE¶

‡*Istituto Regina Elena per lo Studio e la Cura dei Tumori, Roma, Italy,*
§*Service of Cell Proliferation, Istituto Medico Scientifico, Roma, Italy,*
||*Department of Oncology, Istituto Medico Scientifico, Roma, Italy*
¶*Consiglio Nazionale delle Ricerche, Roma, Italy*

Abstract—*The inhibitory effect of vincristine (VCR) on cell division was studied* in vivo *on two squamous cell carcinomas of head and neck. One milligram of VCR was pulse injected in the external carotid artery, followed by a continuous* 20 hr *infusion of another* mg *of VCR. A biopsy in three different areas of the tumor was taken at* 0, 7, 20, 26, 32, 45 *and* 62 hr *after the onset of the experiment. Histologic sections were prepared, mitotic index (M.I.) was counted and DNA content measured microfluorometrically in the interphases and mitotic cells obtained by "squashing" the biopsy. In one case the M.I., which varied in different areas of the tumor, increased during the treatment, remaining significantly higher than the control value until* 42 hr *after the end of the infusion. In the other case at this same time the M.I. restored the initial value. The blocking effect was associated to an alteration in the mitotic cells characterized by the assembling of chromosomes in the middle of the cell and by the disappearance of telophases and anaphases. The metaphase arrest of most of the cycling cells was confirmed by the DNA spectra of the mitotic cells which showed the disappearance of cells with DNA content corresponding to the telophase amount. The relatively small number of cells arrested and the protracted release of the block suggest that a synchronization useful for therapeutic purposes could not be expected in this type of tumors.*

INTRODUCTION

THE ALKALOID VCR is a mitotic inhibitor for several cell types *in vivo* and *in vitro* with a blocking effect in the metaphase [1–3]. The mitotic action is not the only effect of this drug, other reports suggesting also inhibition of the RNA, protein and DNA synthesis [4–8]. Recently the interest for this drug developed towards its potential synchronizing effect, the aim being to accumulate cells in the most sensitive phase(s) of the cycle and then properly scheduling the administration of other cytotoxic agents [9, 10]. A number of clinical trials have been carried out according to these principles, that is, the administration of VCR was followed at different time intervals by, e.g. bleomycin or cyclophosphamide [11–14].

The purpose of this study was to verify by means of morphological analysis, M.I. evaluation and measurement of nuclear DNA content, the mitotic action exerted by VCR on squamous cell carcinoma of head and neck in man. The nuclear DNA content was measured on single cell "squashed" on slides in order to identify the mitotic figures and the cancer cells. On the other hand, an accurate discrimination of the various cells is advisable for the proper interpretation of the results obtained in solid tumors by automated flow cytometry [15–19].

MATERIALS AND METHODS

In two cases with extended (T_3N_2–T_4N_2) head and neck carcinoma (one in the hard palate and the other in the temporal region), 1 mg VCR was pulse injected through a catheter inserted in the external carotid artery via the superficial temporal artery. Then, another milligram of VCR, diluted

Accepted 2 May 1978.
*Presented in part at the III International Symposium on Pulse-Cytophotometry Wien Austria—April 1, 2/1977.
†Financed by the Stefano Siglienti Fund and the generous support of Mrs. Loredana Shenker.
Reprints requests to: Carlo Nervi M.D. Head Dept. of Oncology, Istituto Medico Scientifico—Clinica Villa Flaminia—via L. Bodio, 58—00191 Roma, Italy.

in 1000 cc dextrose, was infused for 20 hr. The intra-arterial drug administration was chosen to obtain a relatively higher concentration at the tumor level. On the other hand the well known neurotoxic effect of VCR was not expected to be increased in comparison with the general route administration, considering that the external carotid artery supplies the tissues of the maxillofacial structure, without a direct extension to the central nervous system. However, the total amount given, i.e., 2 mg, was kept in the usual range of a single VCR dose (1.4–1.5 mg/M^2). For this reason a half of the total dose was injected while the other half was infused. From a theoretical point of view, the infusion time of the synchronizing agent should last several tens of hours to be at least as long as the mean intermitotic time Because of the absence of data in the literature concerning intra-arterial VCR administration, a compromise between total amount, drug concentration and, consequently, infusion time, had to be found. However, a one day infusion time could exploit a stathmokinetic effect on a larger amount of cycling cells than the pulse injection modality. A biopsy specimen was taken, when possible, in three different areas of the tumor three times a day during the first (0, 7, 20 hr) and the second day (26, 32, 45 hr) and 62 hr after the onset of the experiment. Part of the biopsy was fixed in ethanol–acetic acid, Feulgen stained (acid hydrolysis in HCl 3.5 N 17 min at 37°C; basic fucsin 0.05% 1 hr) and then was "squashed" [20] for M.I. evaluation and DNA content measurement. The other part of the biopsy was stained with Mayer hematoxilin and eosin and the sections prepared for histological examination. The M.I. was evaluated and the mitotic phases identified in two slides for each biopsy specimen, on the basis of a minimum of 300 mitotic or 3000 interphase cells. The DNA content of 250–300 mitotic cells in the same scanned area was also measured by means of a Leitz MPV–I cytofluorometer. The fluorescence of the DNA-dye complex was excited by a narrow band interference filter at 480 nm and measured with a barrier filter with the edge at 665 nm for minimizing inner filter effect. Under these conditions, the maximum optical extinction of the nuclei remains below 0.1, so that there is a good proportionality between dye concentration and fluorescence output [21]. The DNA values were normalized to the mean DNA content of 30–40 lymphocytes found in the same measured area. The telophases were measured as single pole so that their relative DNA content is referred to half of the metaphase amount.

RESULTS

Case 1 (*D.F.*)

This tumor was classified histologically as a grade III, moderately indifferentiated carcinoma, composed of a quite homogeneous cell population. The histological picture changed after at least 7 hr after the onset of the experiment, because about 60% of all the mitoses appeared altered being characterized by the assembling of chromosomes in the middle of the cell [22] (Fig. 1). This finding was to be expected because of the specific action of the drug on the mitotic spindle [23, 24]. The predominance of abnormal mitoses lasted until 32 hr after the end of the infusion, but in specimens taken at 45 and 62 hr the fraction of altered mitoses was reduced to about 10%.

The values of the M.I. are reported in Table 1. A chi square test was applied to verify the homogeneity of the M.I. values between slides and specimens. In most instances the test gave significant results either before, or during, and after the treat-

Table 1. *Case* 1. *Values of mitotic index* ± *S.D.* (%)

Time (hr)	Specimen	Slide (A)	Slide (B)
0	0a	1.31 ± 0.13	1.35 ± 0.14
	0b	1.45 ± 0.14	1.40 ± 0.20
	0c	2.47 ± 0.19	2.19 ± 0.24
7	1a	4.71 ± 0.23	4.65 ± 0.24
	1b	6.86 ± 0.29	4.81 ± 0.32
	1c	5.53 ± 0.39	5.82 ± 0.38
20	2a	5.18 ± 0.24	9.68 ± 0.30
	2b	4.05 ± 0.24	2.99 ± 0.23
	2c	5.27 ± 0.39	10.23 ± 0.54
26	3a	4.08 ± 0.22	4.15 ± 0.30
	3b	12.98 ± 0.40	6.00 ± 0.35
	3c	7.67 ± 0.55	4.01 ± 0.28
32	4a	8.29 ± 0.41	5.86 ± 0.29
	4b	5.87 ± 0.41	3.95 ± 0.25
	4c	6.60 ± 0.43	6.64 ± 0.53
45	5a	4.62 ± 0.37	8.65 ± 0.57
62	6a	4.73 ± 0.33	6.98 ± 0.32

M.I. (percent values) in multiple specimens and slides at various times with their standard deviation.

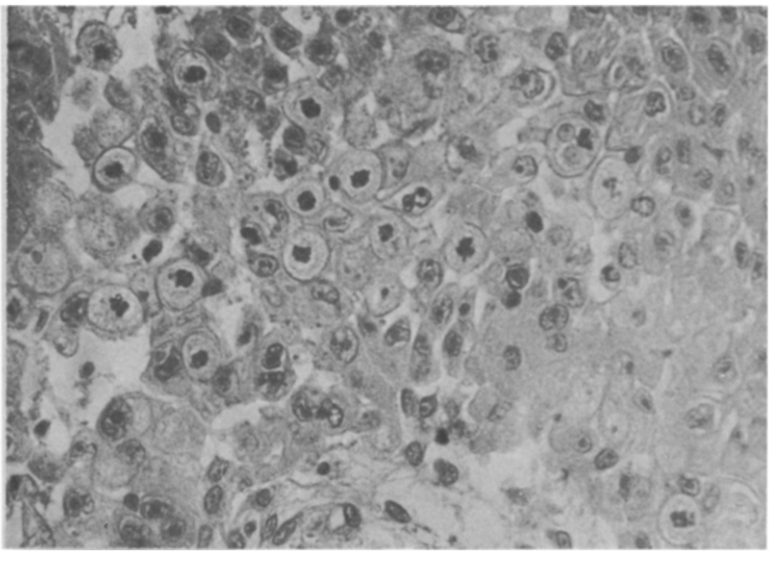

Fig. 1. *Case* 1. *Hematoxilin–eosin stained section at* 20 hr. *Several altered mitotic cells can be observed.*

ment. Therefore, the mean values for each time were not calculated. However, the single values of M.I. during and after the treatment show clearly an upwards tendency.

At time 0, the DNA content of telophases and metaphases was mainly confined to two peaks corresponding to the lymphocytes value and twice as much, respectively (Fig. 2). In the points at 7, 20 and 26 hr the DNA spectra showed the almost complete disappearance of the first peak, irrespectively of the M.I. value (Fig. 3). In the biopsy specimen after 32, 45 and 62 hr, even though the general pattern of DNA distribution is substantially unchanged, there is a small increase of the first peak, likely because of an initial release of the blocking effect (Fig. 4). The identification of mitotic phases allowed the evaluation of their relative frequencies. The mean values for each time are reported in Fig. 5, which summarizes the main features of the experiment and is in agreement with the previous observation, that is, starting at 7 hr, almost all mitoses are found in metaphase.

The DNA content of interphase cells was also measured, but, when comparing the histograms of two biopsy specimens taken at the same time, the chi square test [25] indicates a statistically significant difference at 1% level. This finding was observed either in the specimen at 0 hr or in the following points.

Case 2 (*A.C.*)

The histological picture of this tumor showed a slightly more differentiated structure. Again at 7 hr about 50% of all mitoses appeared altered. However, already at 26 hr the fraction of altered metaphases is reduced to 30%. Starting from 45 hr no more abnormal metaphases could be observed and anaphases and telophases raised from about 3 to 15%.

Also in this case the M.I. value showed large fluctuations either before or during and after treatment (Table 2). However, the increase of the M.I. lasted only 32 hr, compared to the 60 hr of case 1. In the control specimens, the telophases and meta-

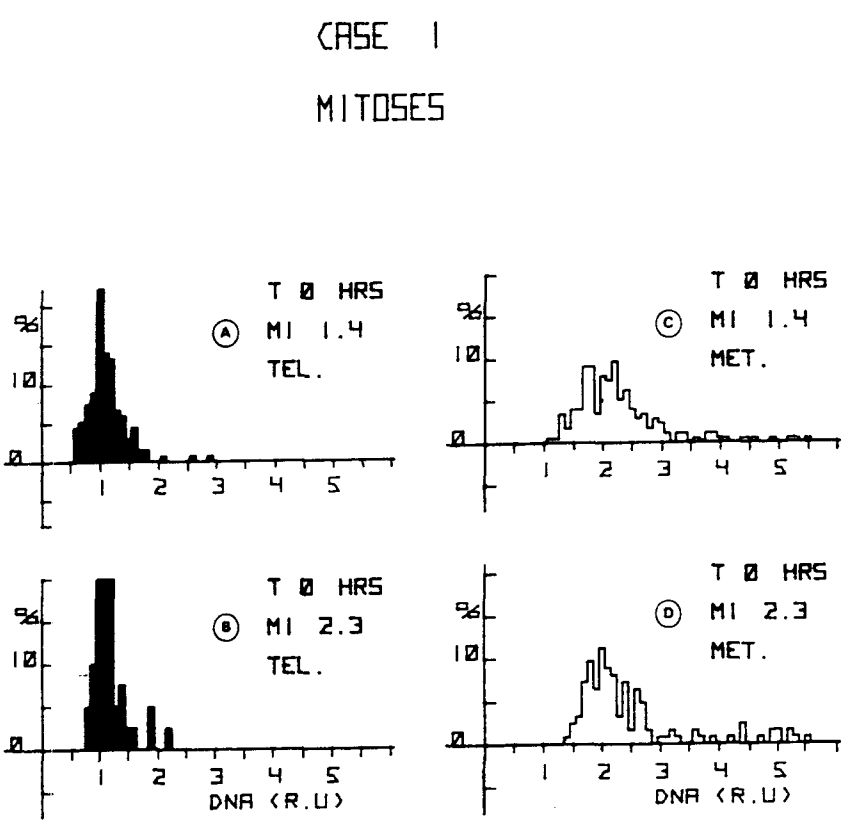

Fig. 2. DNA content of mitoses in case 1 at time 0. The distributions are given in two areas of the tumor characterized by different values of M.I. (R.U.: relative unit of DNA content normalized to mean lymphocytes value).

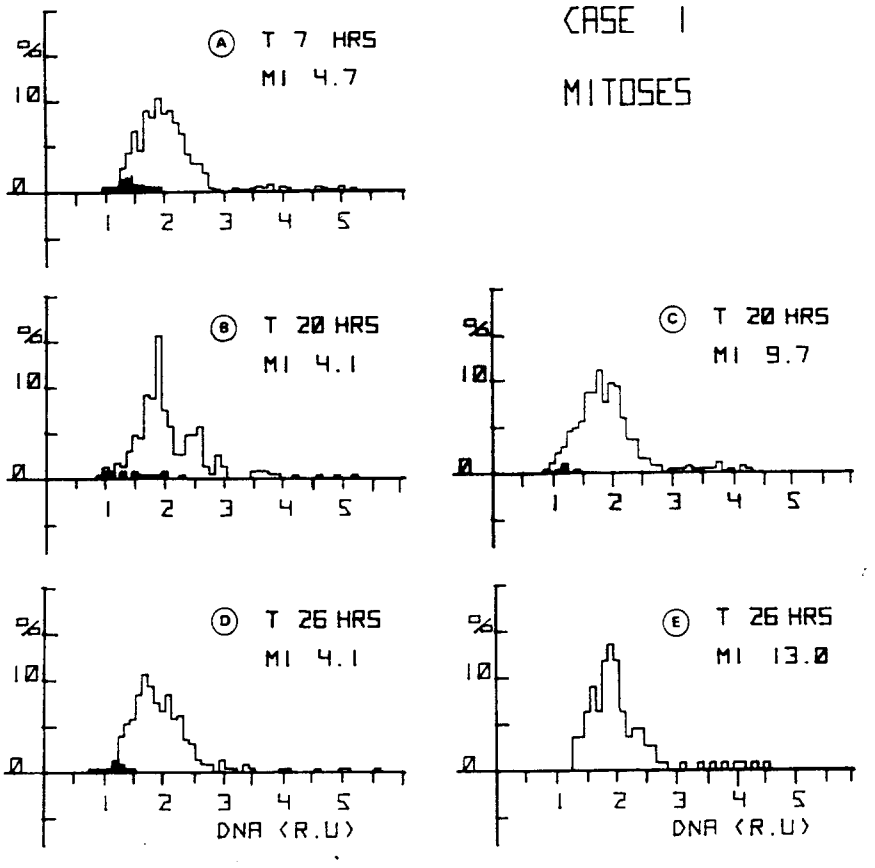

Fig. 3. Case 1. DNA content of metaphases (white histograms) and telophases (dark). At time 20 and 26 hr measurements were taken in specimens with low (B, D) and high (C, E) M.I.

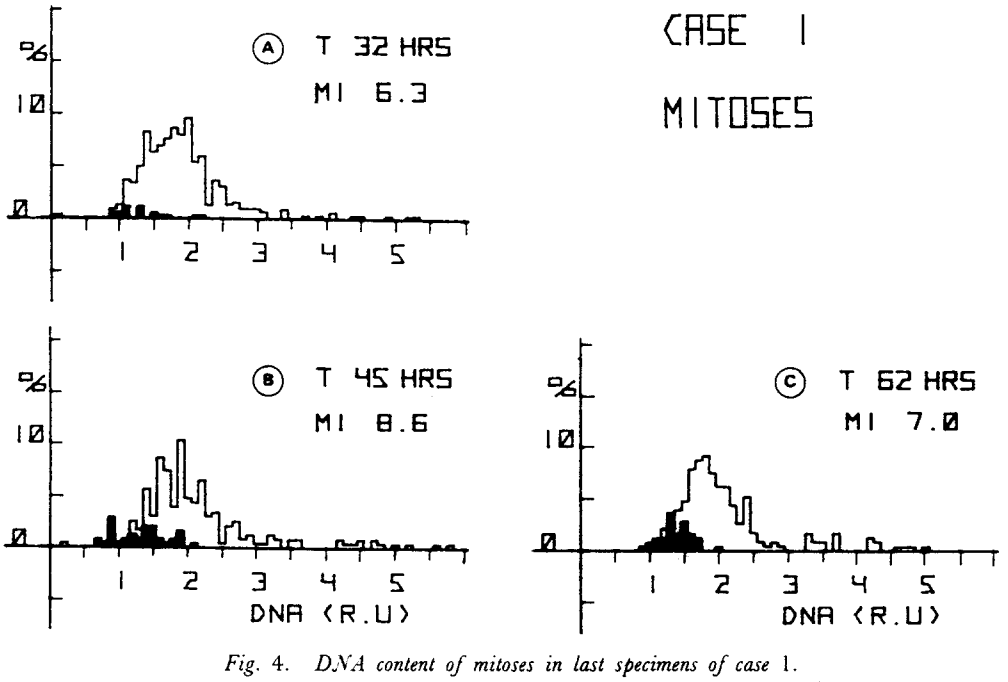

Fig. 4. DNA content of mitoses in last specimens of case 1. Dark histograms are telophases, metaphases are in white.

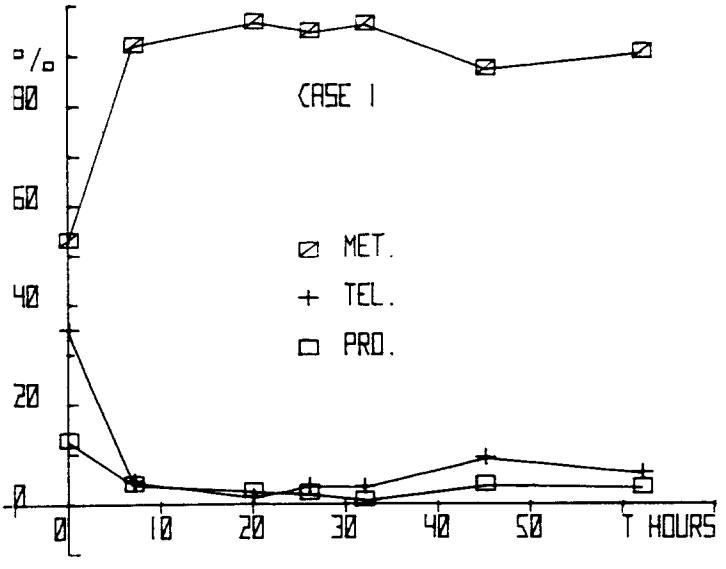

Fig. 5. Case 1. Plot of the relative frequencies of mitotic phases normalized to the total number of mitoses.

Table 2. Case 2. Values of mitotic index ± S.D. (%)

Time (hr)	Specimen	Slide (A)	Slide (B)
0	0a	0.98 ± 0.18	1.33 ± 0.18
	0b	1.48 ± 0.19	1.77 ± 0.29
	0c	0.68 ± 0.16	1.32 ± 0.28
7	1a	5.04 ± 0.31	6.93 ± 0.48
	1b	5.10 ± 0.36	4.12 ± 0.50
20	2a	6.91 ± 0.37	10.56 ± 0.51
	2b	7.29 ± 0.45	6.00 ± 0.33
26	3a	4.20 ± 0.43	6.50 ± 0.39
32	4a	9.04 ± 0.45	7.06 ± 0.59
45	5a	1.89 ± 0.24	1.81 ± 0.36
62	6a	0.27 ± 0.09	0.60 ± 0.21

M.I. (percent values) in multiple specimens and slides at various times with their standard deviation.

phases were confined to two peaks with DNA content slightly higher than the lymphocytes value (Fig. 6 A, B). At 20 hr, no mitoses with DNA corresponding to the first peak could be found. At 26 hr, a small number of nuclei belonged to the first peak (Fig. 6 C, D). In the last specimens, even though the low amount of mitotic figures observed did not permit the construction of satisfactory histograms, 19% of mitotic cells with DNA content corresponding to telophases was found. The plot of the mitotic phases (Fig. 7) showed a gradual return of the fraction of mitoses to the pre-treatment level.

DISCUSSION

The aim of synchronizing procedures is that most of the cells will contemporaneously cross a definite phase of the division cycle. This result can be attained by reversibly blocking the cells in a phase, e.g., mitosis, and then releasing the block in order to obtain a relatively uniform recruitment of the cells into the cycle [26]. Such an approach should make it theoretically possible to take advantage of kinetic differences in the cycle progression between tumor and normal cells. Obviously enough, the drug concentration within the tumor should be kept at a sufficient level for a time at least comparable to the cells cycle times. In our experiment these conditions were approached by employing a continuous regional infusion of the drug, so that a concentration effective for a rather prolonged period of time could be obtained. The efficacy and limits of this procedure were checked by using different complementary methods. In fact, because of its intrinsic variability, the M.I. has only a limited relevance in assessing the action of the drug, whereas the DNA spectra of the mitotic figures clearly demonstrate the presence of the block also in tumor areas where the M.I. showed only small increments. This is evident in Case 1 (Fig. 3 panels B, C and D, E) where more than 90% of the mitoses are metaphases either in the areas where the M.I. is 4.1 and 9.7% or 4.1 and 13% respectively. Therefore,

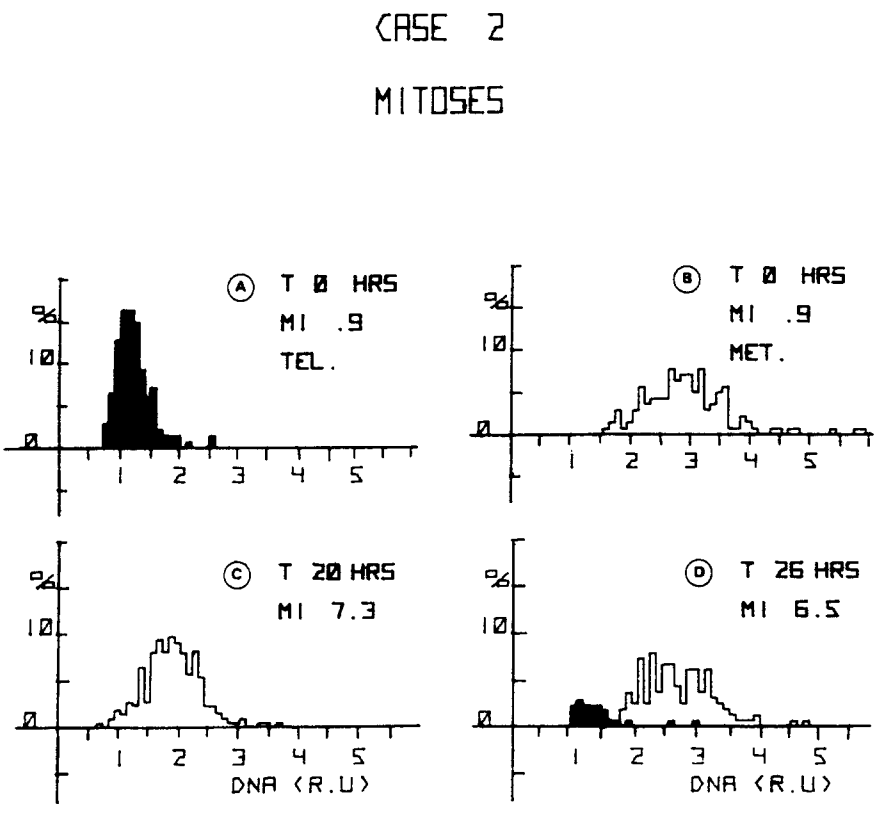

Fig. 6. DNA content of mitoses in case 2 ar various times. Dark histograms, telophases, metaphases in white.

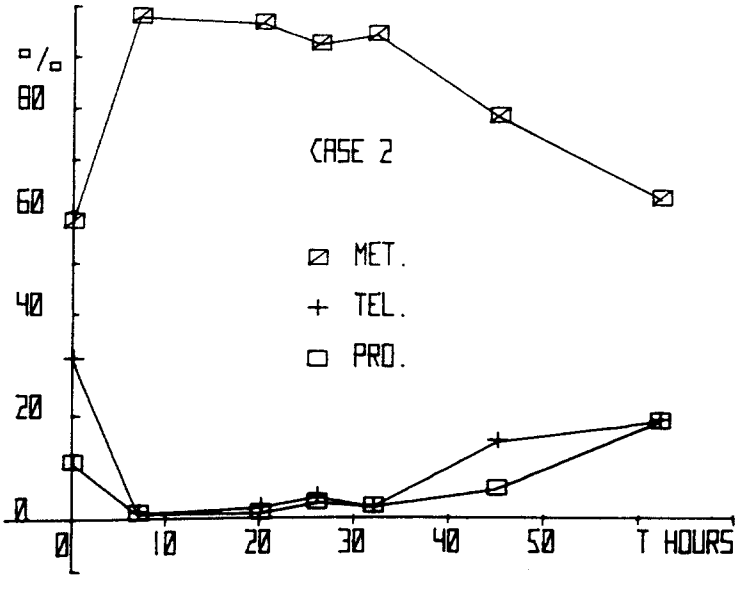

Fig. 7. Case 2. Plot of relative frequencies of mitotic phases, normalized to the total number of mitoses.

the fluctuations of M.I. in this type of tumor should be more likely ascribed to local variation in the proliferative activity rather than to a failure of the drug action. Beside, the DNA measurements confirmed quantitatively that altered mitotic cells are actually cells arrested in metaphase. Concerning the duration of the block, in case 1 it was protracted up to 42 hr after the end of VCR infusion. It seems, therefore, that the drug effect, which results in a metaphase arrest, is taking place also in other phases of the cycle. This was recently demonstrated by the double labelling method

on jejunal crypt cells of the mouse [27]. It does not seem unlikely that the protracted duration of the block should be ascribed to a similar mechanism of action. According to this hypothesis despite the duration of VCR infusion is only a fraction of typical cell cycle time, i.e., 60–70 hr, the values of M.I. (5–9%) in the specimens taken at 62 hr should give an approximate, even though underestimated, size of the proliferating population. On the contrary, in case 2, the duration of the block does not last much longer than the period of infusion. The reasons for this phenomenon could be related either to some estrinsic effect i.e., minor local drug concentration due to unpaired vascularization, or to intrinsic causes, i.e., shorter cycle times. The gradual release of the block, while confirming some possible action in cycle phases other than mitosis, indicates that a sharp synchronization could hardly be obtained. It was not possible to determine whether the normal metaphases and telophases appearing after the blocking action of the VCR are the same cells arrested by the drug or new mitotic figures unaffected by VCR. The DNA spectra of the interphase cells were unable to reveal a synchronizing effect because of the already mentioned statistically significant differences of histograms in different specimens at the same time, likely due to zonal proliferative inhomogeneities.

In conclusion, the *in vivo* analysis of the mitotic effect of VCR suggests:

(a) a blocking action, whose duration depends on each particular tumor, can be attained.

(b) the M.I. value is not a reliable predictor of the blocking action because of its widespread fluctuations.

(c) the arrest affects only a limited proportion of cells, so that a true synchronizing effect is by no means easy to be detected.

(d) synchronization relies on reversibility of the block. Even though, in our cases, this question was not answered, there is some evidence that blocked metaphases are permanently damaged [27].

(e) assuming that sampling and preparative problems are solved, flow cytometry could be an essential help in assessing the presence of synchronously cycling cells.

(f) in view of the difficulties in preestablishing the block duration and the modalities of the block release, a clinically useful synchronization seems difficult to be obtained.

(g) the relevance of kinetic procedures in terms of therapeutic gain seems to be limited in cases similar to the present ones, mainly because of the small fraction of proliferating cells.

REFERENCES

1. G. Cardinali and M. A. Enein, Studies on the antimitotic activity of leurocristine (VCR). *Blood* **21,** 102 (1963).
2. E. Frei, J. Whang, R. B. Scogging, E. J. van Scott, D. P. Rall and M. Ben, The stathmokinetic effect of vincristine. *Cancer Res.* **24,** 1918 (1964).
3. P. George, L. G. Journey and M. N. Goldstein, Effect of vincristine on the fine structure of HeLa cells during mitosis. *J. nat. Cancer Inst.* **35,** 355 (1965).
4. W. A. Creasey, Modifications in biochemical pathways produced by the vinca alkaloids. *Cancer Chemother. Rep.* **52,** 501 (1968).
5. W. A. Creasey and M. E. Markiv, Biochemical effects of the vinca alkaloids. II. A comparison of the effects of colchicine, vinblastine and vincristine on the synthesis of ribonucleic acids in Ehrlich ascites carcinoma cells. *Biochim. biophys. Acta. (Amst.)* **87,** 601 (1964).
6. H. Madoc-Jones and F. Mauro, Interphase action of VLB and VCR: differences in their lethal action through the mitotic cycle of cultured mammalian cells. *J. Cell Physiol.* **72,** 185 (1969).
7. M. Cline, Effect of VCR on synthesis of RNA and protein in leukaemic leukocytes. *Brit. J. Haematol.* **14,** 21 (1968).
8. H. Madoc-Jones, A review of the action of various chemotherapeutic agents on mammalian cells in the different phases of the cell generation cycle: possible clinical implication. In *Aktuelle Probleme der Therapie Maligner Tumoren.* p. 186. Georg Thieme Verlag, Stuttgart (1973).
9. E. Frei, Combination cancer therapy. Presidential address. *Cancer Res.* **32,** 2593 (1972).
10. B. C. Lampkin, N. B. McWilliams, A. M. Mauer, H. C. Flessa, D. A. Hake and V. Fisher, Manipulation of the mitotic cycle in the treatment of acute myelogeneous leukemia. *Brit. J. Haematol.* **32,** 25 (1976).

11. L. M. VAN PUTTEN, H. J. KEIZER and J. H. MULDER, Perspectives in cancer research. Synchronization in tumor chemotherapy. *Europ. J. Cancer* **12,** 79 (1976).
12. P. POUILLART, R. WEINER, J. L. MISSET and L. SCHWARZENBERG, Combination chemotherapy based on model of recruitment by partial synchronization. *Proc. Amer. Ass. Cancer Res.* **14,** 115 (1973).
13. R. B. LIVINGSTON, G. P. BODEY, J. A. GOTTLIEB and E. FREI, Kinetic scheduling of vincristine (NSC-123066) in patients with lung cancer and other malignant tumors. *Cancer chemother. Rep.* **57,** 219 (1973).
14. A. SOWISTKY, F. DEPOSITO, C. TREAT *et al.*, Vincristine and cyclophosphamide therapy in generalized neuroblastoma. *Amer. J. Dis. Child.* **119,** 308 (1970).
15. H. O. KLEIN, D. ADLER, M. DOERING, P. J. KLEIN and K. J. LENNARTZ, Investigations on pharmacologic induction of partial synchronization of tumor cell proliferation: its relevance for cytostatic therapy. *Cancer Treat. Rep.* **60,** 1959 (1976).
16. E. SEVERIN, Comparative microfluorometric investigation by MPV and IPC on liver cell and synchronized tumor cells. In *First International Symposium on Pulse Cytophotometry, Nijemegen* 1974. p. 42. European Press, Gent. (1975).
17. H. A. CRISSMAN and R. A. TOBEY, Cell cycle analysis in 20 min. *Science* **184,** 1297 (1974).
18. J. A. STEINKAMP, M. J. FULWYLER, J. R. COULTER, R. D. HIEBERT, J. L. HORNEY and P. F. MULLANEY, A new multiparameter separator for microscopic particles and biological cells. *Rev. Sci. Instr.* **44,** 1301 (1973).
19. M. A. VAN DILLA, T. T. TRUJILLO and P. F. MULLANEY, Cell microfluorometry: a method for rapid fluorescence measurements. *Science* **163,** 1213 (1969).
20. M. FRIEDMAN, C. NERVI, C. CASALE, G. STARACE, G. ARCANGELI, G. PACE and E. ZIPARO, Significance of growth rates, cell kinetics and histology in the irradiation and chemotherapy of squamous cell carcinoma of the mouth. *Cancer (Philad.)* **31,** 10 (1973).
21. G. PRENNA, G. MAZZINI and S. COVA, Methodological and instrumentational aspect of cytofluorometry. *Histochem. J.* **6,** 259 (1974).
22. W. DAHL NORBØ, R. OFTEBRO, E. O. PETTERSON and T. BRUSTAD, Inhibitory and cytotoxic effect of oncovin (vincristine sulfate) on cells of human line NHIK 3025. *Cancer Res.* **36,** 3101 (1976).
23. R. H. HIMES, R. N. KERSEY, HELLER-I. BETTINGER and F. E. SANSON, Action of the vinca alkaloids vincristine, vinblastine and desacetyl vinblastine amide on microtubules *in vitro*. *Cancer Res.* **36,** 3798 (1976).
24. L. J. JOURNEY, J. BURDMAN and P. GEORGE, Ultrastructural studies on tissue culture cells treated with vincristine (NSC-67574). *Cancer Chemother. Rep.* **52,** 509 (1968).
25. W. T. EADIE, D. DRIJARD, F. E. JAMES, M. ROOS and B. SADOULET, *Statistical Methods in Experimental Physics* p. 279. North Holland, Amsterdam (1977).
26. C. NICOLINI, The principles and methods of cell synchronization in cancer chemotherapy. *Biochim. biophys. Acta (Amst.)* **458,** 243 (1976).
27. W. JELLINGHAUS, B. SCHULTZE and W. MAURER, The effect of vincristine on mouse jejunal crypt cells of differing cell age: double labelling autoradiographic studies using ^{3}H and ^{14}C TdR. *Cell Tiss. Kinet.* **10,** 147 (1977).