Eur J Cancer Clin Oncol, Vol. 19, No. 8, pp. 1113–1119, 1983.
Printed in Great Britain.

0277-5379/83$3.00+0.00
Pergamon Press Ltd.

The Effect of *N*-Formylleurosine on DNA Synthesis of Ehrlich Ascites Tumor

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Abstract—*Some of the vinca alkaloids are well known and widely used in clinical practice, in spite of their numerous side-effects. For the elimination of untoward side-effects semi-synthetic alkaloids have been produced. This work reports on* N-*formylleurosine* (N-*F-Leu*), *one of these agents. Its antitumour activity is, in many respects, similar to that of other, presently used vinca alkaloids; it causes metaphase block, the appearance of multinucleated cells and polyploidization. In addition to these effects,* N-*F-Leu induces significant new phenomena, namely, changes in the activity of thymidine kinase, a key enzyme of DNA synthesis as well as in thymidine incorporation, and reduces the proportion of cells in S phase in a relatively short period of time after treatment:* 24–29 hr. *Based on these observations, the re-evaluation of the mechanism of action of vinca alkaloids has become possible.*

INTRODUCTION

VINCA alkaloids belong to the group of the 'spindle poisons'. Their most characteristic effect is arresting cells in metaphase [1–3]. Drug effect may manifest itself in the prevention of the mitotic spindle formation [4]. Structural changes of the spindle result in mitotic disturbances, e.g. in the dispersion of chromosomes in the cytoplasm, leading to the appearance of multinucleated cells.

Among the various vinca alkaloids, vincristine (VCR) and vinblastine (VLB) have been used for inhibiting tumour growth. Both compounds have, however, toxic side-effects.

Biosynthetic processes are adversely affected by vinca alkaloids. For instance, neurotoxicity of VCR is due to its depressent effect on phospholipid biosynthesis, resulting in the block of axonal transport [5, 6]. Changes induced by VCR and VLB in the synthesis of DNA and of other macromolecules have also been reported [7–13]. *N*-Formylleurosine (*N*-F-Leu), another alkaloid of *Vinca rosea*, was found to be less toxic than VLB or VCR, both *in vivo* and *in vitro* [14], and devoid of neurotoxicity.

The tumour inhibitory effect *N*-F-Leu is similar to that of VCR. It is, however, 7 times less toxic than VCR, 4.5 times less toxic than VLB and shows no signs of neurotoxicity [15]. A clinical trial of *N*-F-Leu was reported by Eckhardt [16].

Accepted 26 February 1983

In this study we wished to examine whether *N*-F-Leu (1) induces metaphase block; (2) causes the appearance of multinucleated cells; (3) changes the amount of the DNA in the tumour cells; (4) affects DNA synthesis.

MATERIALS AND METHODS

Experiments were carried out on Ehrlich ascites tumour cells maintained in Swiss-H-Riop outbred mice. *N*-Formylleurosine (Gedeon Richter Pharmaceutical Works, Ltd.) was given in different single intraperitoneal doses on the 6th day after transplantation. In each experiment 4 animals were treated and their tumour cells pooled. Samples were taken at different intervals from the same treated and control animals to examine drug-induced alterations.

Morphological studies

Dosage: 2, 5, 10 and 15 mg/kg respectively. Smears were stained with acridine orange according to Armstrong [17] to detect drug-induced morphological changes. Points of time examined: 3, 6, 24, 27, 29, 48, 72 hr after treatment. Smears were evaluated under a Leitz-Orthoplan fluorescent microscope.

Quantitative cytochemical studies

In these examinations a dose of 10 mg/kg was used. Cytophotometric measurements were done 24, 27, 29 and 48 hr after treatment.

Changes in the DNA content of tumour cell

nuclei were determined with a Barr and Stroud [18]-type scanning and integrating cytophotometer. Staining procedure was as follows: smears of tumour cells were fixed for 15 min in Carnoy solution and washed for 4 × 1 min in distilled water. Then Feulgen's staining reaction with Schiff reagent was performed as modified by Lillie [19]. Preparations were mounted with Canada balsam. At each point of time the DNA content of 200 tumour cells was determined at 550 nm wavelength and the values were expressed in arbitrary units.

Biochemical studies

Examinations were performed at 24, 27, 29 and 48 hr after the administration of 10 mg/kg *N*-F-Leu. An approximately 10-fold dilution of the ascites was made by a 1:4 mixture of sodium citrate (3.3%) and Hanks' solution. Cells were washed twice (at 4°C) and then resuspended in Hanks' solution. The cell suspension was divided into two parts, one for thymidine kinase assay and the other for thymidine incorporation measurements.

Thymidine kinase assay

A suspension of approximately 10^7 cells/ml was sonicated with an MSE 100 Watt Ultrasonic Disintegrator at maximum output for 3 × 30 sec at 0°C. The solution was then centrifuged in a Janetzky VAC 601 at 105,000 *g* for 90 min at 4°C. Enzyme activity in the supernatant was measured as described by Szikla *et al.* [20].

Thymidine incorporation

Washed tumour cells were incubated in Hanks' solution (10^7 cells/ml) containing 20 amino acids (0.1 mM of each), 0.3% glucose, 0.145 mM thymidine and 244.2 kBq [^{3}H]-thymidine (854.7 GBq/mM/ml) at 37°C.

Aliquots were taken at 20, 40 and 60 min respectively of the incubation period, and incorporation was stopped in 0°C Hanks' solution containing 1 mg/ml thymidine. Cells were washed twice with the same solution, precipitated in 5 ml 0.7 N perchloric acid (PCA) and centrifuged at 5000 *g*.

The precipitate was washed twice in 0.7 N PCA solution, once in ethanol (96%), then in a mixture of ether–ethanol (3:1 v/v) and finally in ether. The ether-dried powder was dissolved in formic acid (2–3 ml).

Radioactivity was measured by liquid scintillation in a mixture of 10 ml scintillation fluid (PPO, POPOP, toluol) and 2 ml absolute ethanol. Protein content was measured after neutralizing the formic acid solution with NaOH by the method of Hartree [21].

Autoradiography

The mice, treated with 10 mg/kg *N*-F-Leu, were injected with 37 kBq/g [^{3}H]-thymidine ([^{3}H]-TdR) i.p. 24, 27, 29 and 48 hr after treatment. The animals were killed 30 min later. The ascites cells were aspirated, washed in Hanks' solution (5-fold volume at 0°C) and centrifuged 3 times. The smears were fixed with Carnoy's solution and stained by Feulgen's method. Autoradiographs were prepared using Ilford G-5 liquid emulsion and developed after a 4-day exposure.

RESULTS

N-F-Leu treatment caused mitotic block in a wide dose range (2–15 mg/kg) in Ehrlich ascites tumour cells. Changes in the mitotic count during the first 24 hr were dose-dependent. Nearly all mitotic figures were abnormal, chromosomes were scattered in the cytoplasm and no division of the cytoplasm was observed. The highest mitotic rate by each dose was achieved by 24 hr (Fig. 1). By 27 hr the number of mitoses fell by 50% and remained the same at 29 hr after treatment with 10 mg/kg. At later points of time mitotic rate was similar and showed only minimal changes.

The proportion of multinucleated cells increased gradually and reached a maximum at 48 hr (with 15 mg/kg dose it was as high as 70%).

The effect of the 10-mg/kg dose was further studied by quantitative cytochemical and biochemical methods.

At 24 hr after treatment the majority of tumour cells, as determined by cytophotometry, were still in the 2- to 4-C range, although a small proportion of cells with a higher DNA content were also encountered.

Cells with a DNA content corresponding to 4 C were more numerous than the control. From 27 to 29 hr 2-C cells were further reduced in number, 4-C cells became dominating and cells with 6–8 C were also increased in number. At later points of time polyploidy became more pronounced (Fig. 2).

Thymidine kinase activity of cytosol extracted from untreated tumour cells did not show significant changes during days 5–7 after transplantation: they were, in fact, almost identical with the control values at any point of time.

Cytosol of tumour cells treated with 10 mg/kg *N*-F-Leu exhibited a continuously decreasing enzyme activity until 27 hr (Fig. 3). From 27 to 29 hr, however, there was a rapid rise in enzyme activity. Later it decreased to about half its value, although it was twice as high as that of the control. The rate of thymidine incorporation showed similar changes after *N*-F-Leu treatment.

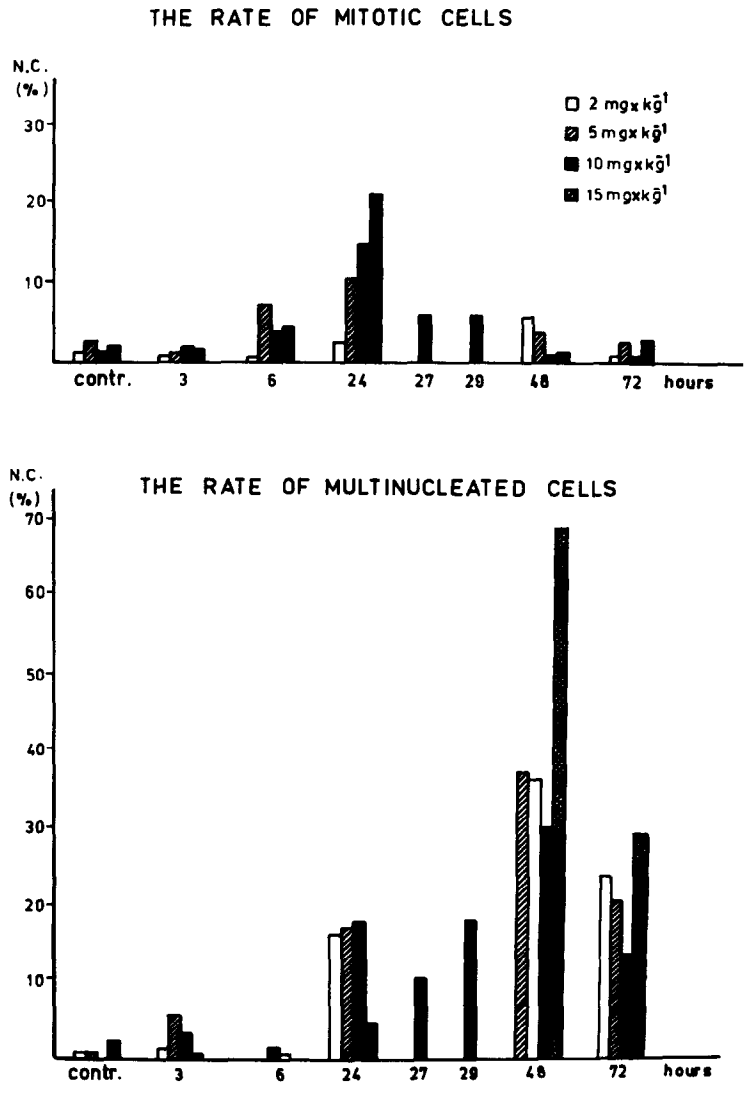

Fig. 1. Treatment with varying doses of N-*F-Leu induced maximum mitotic count* 24 hr *after drug administration. Multinucleated cells appeared in highest number* 48 hr *after treatment. At later points of time the mitotic count was found to diminish to the control level, whereas the number of multinucleated cells remained higher than the control throughout the observation period. At* 27 *and* 29 hr *only the* 10 mg/kg *dose was studied.*

Both thymidine kinase activity and incorporation were continuously decreasing to a minimum of approx. 60% of the control until 27 hr. From 27 to 29 hr, the rate of thymidine incorporation suddenly rose, surpassing the control level. Further significant changes could not be observed until 48 hr (Fig. 4).

Autoradiography showed that the number of labelled cells reduced to half that of the control at 29 hr and increased to near the control level at 48 hr (Fig. 5).

DISCUSSION

The characteristic effect of vinca alkaloids hitherto used in clinical practice is arresting cells in metaphase [2, 3]. This phenomenon is also apparent for *N*-F-Leu whatever dose is applied. The maximal mitotic rate was observed at 24 hr after treatment. By 27 hr, however, the mitotic count had decreased by half and remained low until 29 hr, thereafter reducing to the control level.

Thorough analysis of histograms of the untreated control cells with DNA content in the 2- to 4-C [22] range demonstrated aneuploidy, characteristic of most malignant tumours [23].

The trend of the drug response becomes quite clear when the respective ranges or segments of the histograms are examined separately. By 24 hr after treatment an accumulation of cells with a 4-C DNA content was observed which continued until 27 hr. At 29 hr cells with DNA content higher than 4 C appeared. These polyploid cells

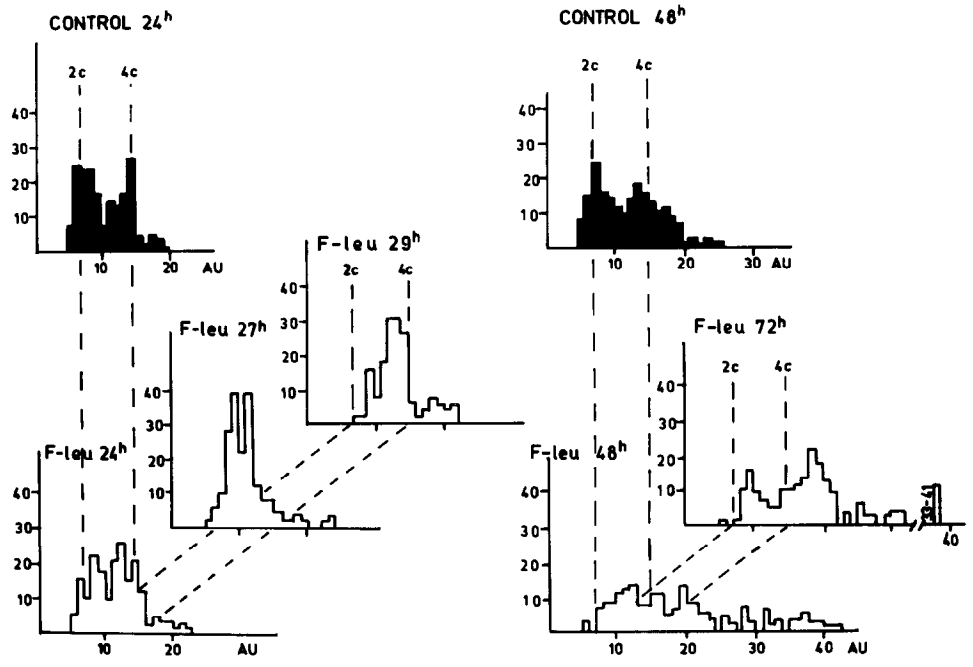

Fig. 2. DNA histograms of N-*F-Leu treatment. Histograms prepared from cytophotometric measurements show that during* 24–27 hr *after treatment the majority of cells have DNA content corresponding to* 2–4 C. *In the course of time less and less cells are found within this range, with an increasing number of cells having a DNA content greater than* 4 C.

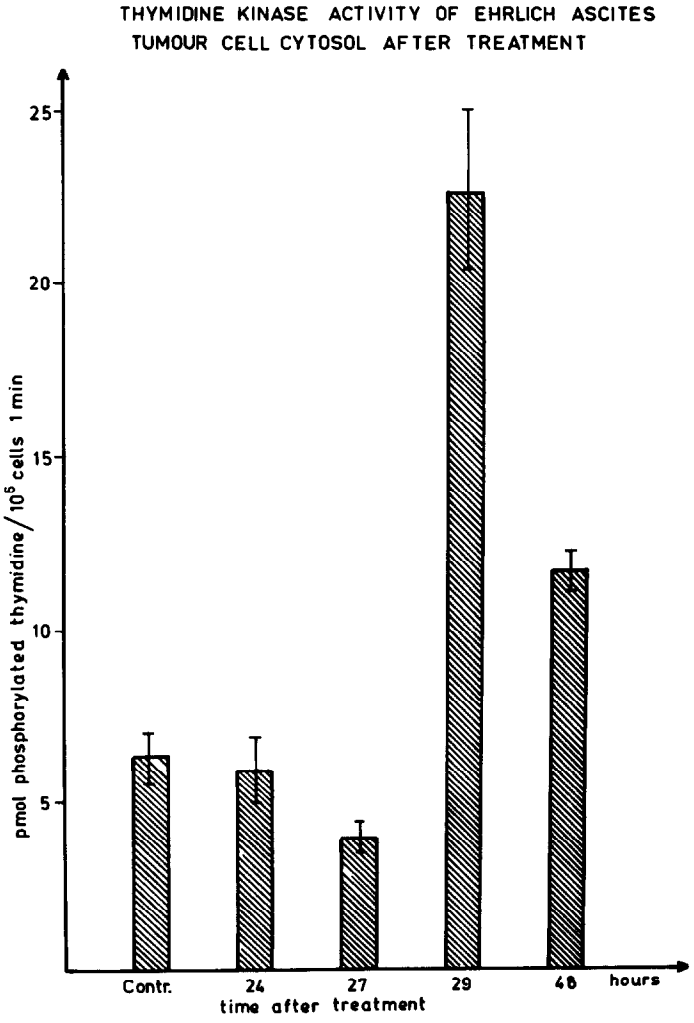

Fig. 3. Changes in the level of thymidine kinase activity of cells after treatment with 10 mg/kg N-*F-Leu. Enzyme activity of the control population showed minimal changes during the observation period. Thymidine kinase activity reduced during* 24–27 hr. *However, it showed an extreme rise by* 29 hr *and remained at a high level even at* 48 hr.

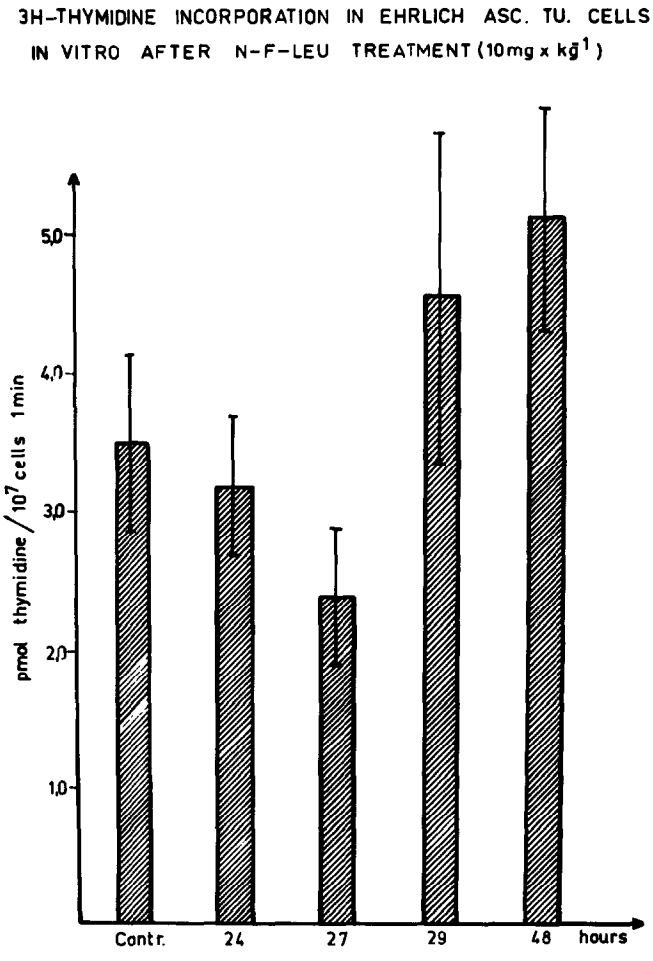

Fig. 4. Thymidine incorporation of tumour cells after treatment with 10 mg/kg N-*F-Leu showed a decrease during* 24–27 hr *and then a rise by* 29 hr. *This rise was, however, not as pronounced as the controls.*

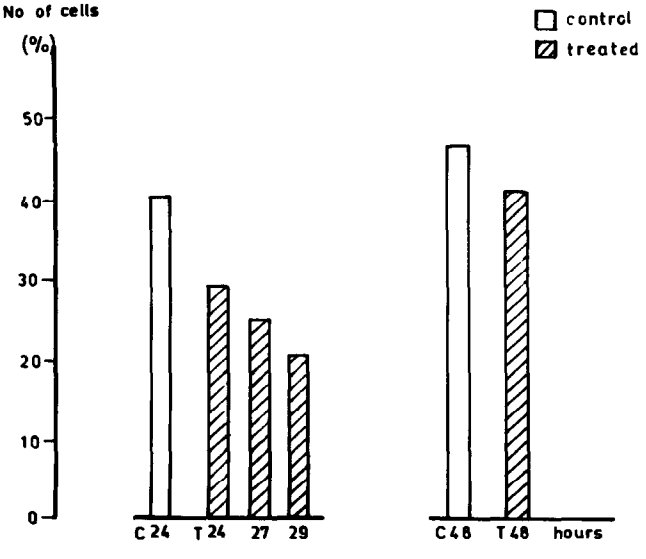

Fig. 5. Percentage of labelled cells at various times after treatment. The number of labelled cells is decreased 29 hr *after treatment with* 10 mg/kg N-*F-Leu, but at* 48 hr *is near the control level.*

were growing in number until 48 hr. The polyploidy induced by other vinca alkaloids has also been reported [24].

Correlation of the above quantitative cytochemical data with results concerning thymidine kinase activity and thymidine incorporation permits the following conclusions.

Cell cycles of the surviving cells are temporarily blocked in metaphase or in G_2, as confirmed by Stöhr and Fischinger [25] in their studies with VCR. Maximum block of the G_2 phase is at 27 hr, as reflected by the accumulation of cells with a 4-C DNA content and the decrease of thymidine kinase activity and incorporation to a minimum. Data obtained at 29 hr suggest that most of the cells are released from this block in a very short time and synthesize DNA intensively. This means that cells doubled their 4-C DNA content, which can be regarded as endoreduplication and resulted in mononuclear polyploid cells [26]. On the other hand, cells released from the metaphase block became multinucleated through attempted mitosis [27]. Formation of multinucleated cells from abnormal mitoses seems to be evident from Fig. 1, where the mitotic peak at 27 hr is reduced and followed by an enormous increase of multinuclear cells by 48 hr.

Impairment of the surviving cells manifests itself in an altered functional state accompanied by the appearance of numerous polyploid cells. Further studies are required to clear up whether this impairment is reversible or not.

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