Interference of Daunomycin with Metabolic Events of the Cell Cycle in Synchronized Cultures of Rat Fibroblasts

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SUMMARY

The influence of daunomycin on nucleic acid synthesis during different stages of the cell cycle has been studied in mammalian cells. Explants from leg muscle of newborn rats were synchronized by two 24-hr treatments with excess thymidine, 16 hr apart. The metabolic activity of DNA and RNA was determined by autoradiographic methods by measuring the incorporation of thymidine-3H, deoxy-cytidine-3H, and uridine-3H into nuclear structures. In control cultures, DNA synthesis shows two distinct waves located in the early and late S phase, the latter being the most sensitive to the inhibiting effect of daunomycin. RNA synthesis takes place in the cells during the entire cell cycle from the end of mitosis to the late prophase, but two well-defined peaks can be recognized: the first in the G2 phase just 1 or 2 hr before the mitotic peak and the second in the middle stages of G1. These RNA syntheses involving both nucleolar and extranucleolar structures are strongly inhibited by daunomycin treatment. The significance of these RNA syntheses in control cells and the relationship between their inhibition and the antimitotic effect of daunomycin are discussed.

INTRODUCTION

Daunomycin is an antibiotic of the anthracycline group isolated from cultures of Streptomyces peucetius (5, 12). This substance consists of a pigmented aglycone (daunomycinone) bound by a glycosidic linkage to an amino sugar (daunosamine) (1, 2). In vitro, daunomycin shows a strong inhibiting effect on the mitotic activity of different types of mammalian cells (10).

The ability of daunomycin to bind to DNA in vitro has been demonstrated by several physicochemical procedures (4, 13, 14). Some of the antimitabolic effects of this drug, such as the inhibition of DNA and RNA synthesis, have also been demonstrated repeatedly (6, 8, 9, 18, 19, 21).

However, the correlation between the antimetabolic effects and the antimitotic activity is not clear at present. In this regard it must be remembered that direct phase-contrast observations showed that daunomycin can arrest the mitotic activity of single cells when added up to 20 min before the prophase stage (10). Furthermore, on HeLa cell cultures and on rat methylcholanthrene sarcoma cells, a remarkable inhibition of the mitotic index takes place with doses of daunomycin, which inhibit only slightly the incorporation of thymidine-3H into DNA (7).

In view of these observations, the possibility of an antimitotic activity of daunomycin unrelated to the effects on DNA and RNA synthesis must also be considered. For further investigation of this problem, a group of experiments on synchronized cultures with the addition of daunomycin during different stages of the cell cycle was carried out. The effects on DNA and RNA synthesis and on the mitotic index were studied.

MATERIALS AND METHODS

Explants from the leg muscle of newborn rats (24 to 48 hr old) were cultivated in Porter flasks in a medium consisting of Hanks' salt solution, human placental serum, and chick embryo extract (4:4:2). After 30-hr growth the cultures were synchronized by two 24-hr treatments with excess thymidine (2 mM), separated by an incubation period of 16 hr (3).

At the end of the treatment with the antibiotic and after exposure to different precursors, the cultures were fixed in Bouin's solution, washed in 5% trichloracetic acid at 4° and in carrier solution, and covered with AR 10 Kodak stripping film as described below (15).

The rates of DNA and RNA synthesis were evaluated by counting the number of reduced silver grains in the whole nucleus and in the nucleolar and extranucleolar structures, respectively. Each value shown in the graph represents the average of counts made on at least 100 cells.

Labeled Substances. These were thymidine-3H (specific activity, 160 mCi/mmmole), 2 μCi/ml culture medium; deoxy-cytidine-3H (specific activity, 500 mCi/mmole), 5 μCi/ml culture medium; and uridine-3H (specific activity, 2.4 Ci/mmole), 5 μCi/ml culture medium.

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RESULTS

Control Cultures

Mitotic Activity. The mitotic index in untreated cultures is about 3%; after synchronization the cell population shows a 1st mitotic peak (14 to 17%) within 6 to 10 hr. The 2nd mitotic peak is not always sharply outlined and the mitotic index is lower than that of the 1st. There is a period of ca. 23 hr between the 2 mitotic peaks, indicating that the length of the cell cycle of rat fibroblasts is the same before and after synchronization (Chart 1).3

Incorporation of Deoxycytidine-3H. After treatment with excess thymidine, the population emerges in the S phase. The use of deoxycytidine-3H clearly shows that at the end of synchronization 90 to 95% of the nuclei are labeled, showing that almost the entire cell population is in the S phase.

The percentage of labeled nuclei remains high until the 4th hr; then it progressively decreases as the cells enter the G2 phase (Chart 2).

The estimation of the nuclear grains demonstrates that the pattern of deoxycytidine-3H incorporation undergoes marked changes during the S phase (Chart 3). Immediately after the end of synchronization, a high degree of nuclear labeling is observed. This value decreases rapidly and then reaches another maximum after 4 to 5 hr. i.e., 3 to 4 hr before the mitotic peak.

This 2nd period of DNA synthesis occurs during the last 2 hr of the S phase.

3The length of the cell cycle and the single phases in an asynchronous population of rat fibroblasts, estimated by a pulse labeling experiment described by Post et al. (16), is as follows: cell cycle, 21 to 23 hr; G2, 2 hr; M 1 hr; S, 8 hr; and G1, 10 to 12 hr.

Chart 1. Mitotic activity of rat fibroblast cultures synchronized by two 24-hr treatments with excess thymidine 16 hr apart.

Chart 2. Mitotic activity and percentage of nuclei labeled with deoxycytidine-3H in synchronized control cultures. Batches of cultures were fixed after 1-hr contact with the labeled precursor.

Incorporation of Uridine-3H. Cells kept in contact with uridine-3H for 1 hr after the end of synchronization show a progressive increase in labeling from 2 to 5 hr when a maximum is observed in both nucleolar and extranucleolar structures. The nuclear labeling then falls to the basic value observed at the beginning of the S phase. In this experiment, the maximum value of the mitotic index (16%) is noted after 6 hr (Chart 4). Maximum RNA synthesis occurs exactly 1 hr before the mitotic peak but drops to much lower values by the time the mitotic activity is at its highest point. In the period of maximum uridine-3H incorporation, the labelings...
The RNA synthesis has also been followed in the G1 phase. The results of the labeling in the nucleolar and extranucleolar regions on incubation of the cultures in the presence of uridine-^3H for 2-hr periods are reported in Chart 5. Uridine-^3H incorporation occurring between the 2 mitotic waves undergoes quite marked variations. In the extranucleolar area of the nucleus an increase in the number of grains is observed from the 15th hr until the 19th hr. This increase is particularly evident at the 15th hr; then the extranucleolar labeling slowly decreases and at the 21st hr the value is similar to that found at the beginning of the G1 phase. More evident changes are observed in the nucleoli, where the nucleolar RNA synthesis is rather low in the 1st hr of the G1 phase, then after 14 hr increases rapidly and reaches a value 3 times higher than at the outset. This rate of production lasts until the 17th hr, then decreases, but does not reach the low values of the early G1 phase. These results have also been confirmed in several experiments.

**Daunomycin-treated Cultures**

**Interference with Mitotic Activity.** Batches of synchronized cultures were treated with daunomycin (0.1 µg/ml) at intervals of 1 hr from the end of synchronization until the onset of mitotic activity. After treatment, the antibiotic was removed and normal culture medium was added. The cultures were fixed after 6, 7, 8, or 9 hr and the mitotic index was determined. As can be observed in Chart 6, in the cultures treated with daunomycin, independent of when treatment is given, the mitotic activity is considerably reduced or completely absent. Because of the binding capacity of daunomycin to DNA the drug cannot be removed from the nuclear structures simply by washing the cell cultures. It must be expected, therefore, that its activity persists for many hours after its removal from the medium.

That daunomycin effectively prevents the mitotic division of cells is shown from the results of the experiment reported in Chart 7, where daunomycin in 2 different concentrations was added to the medium for 1 hr, together with VLB (0.5 µg/ml). In this way the total number of cells arrested in metaphase (stathmokinetic index) could be evaluated.

**Interference with DNA Synthesis.** Interference with deoxycytidine-^3H incorporation caused by daunomycin varies widely with drug concentration and with time of administration. Treatment with 0.5 µg/ml in the early S phase does not affect the DNA metabolic pattern (Chart 8). Treatment with lower doses (e.g., 0.1 µg/ml) over the corresponding period induces a clear and significant increase in nuclear labeling. In other words, the nuclear labeling does not decrease as in the controls, but maintains a high value for 2 hr. The treatment during the last part of the S phase causes a reduction in deoxycytidine-^3H incorporation which is proportional to the concentration of the drug.

**Interference with RNA Synthesis.** The influence of

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The abbreviation used is: VLB, vinblastine.
Chart 5. Mitotic activity and uridine-$^3$H incorporation in synchronized control cultures after 2-hr contact with the labeled precursor.

Chart 6. Interference of daunomycin (Da) with mitotic activity in synchronized cultures. Groups of cultures were treated for successive 1-hr periods and washed and incubated in normal medium. Batches of cultures from each group were fixed during the 6th, 7th, 8th, and 9th hr. *Thick lines*, time of antibiotic treatments.

Daunomycin on RNA synthesis was followed during all phases of the cell cycle.

For the purpose of analyzing to what extent daunomycin treatment during the $G_1$ phase interferes with RNA synthesis, a group of cultures was treated every 2 hr with uridine-$^3$H and daunomycin from the 9th to the 21st hr after the end of synchronization, and then immediately fixed. A nearly complete inhibition of incorporation of uridine-$^3$H into the nucleolus was observed (Chart 9). The degree of inhibition is highest halfway through the $G_1$ phase when a peak of nucleolar RNA synthesis occurs in control cells (see Chart 5). At the same time extranucleolar RNA synthesis is also inhibited but not as markedly as the nucleolar synthesis.

Another group of cultures was treated at corresponding periods and exposed to thymidine-$^3$H from the 21st to the 29th hr. The inhibition of DNA synthesis is first noticed in the cultures treated from the 11th to the 13th hr and becomes pronounced in the cells treated from the 13th hr to the 17th hr, *i.e.*, the same period as that showing strong inhibition of nucleolar and extranucleolar RNA synthesis.

Chart 7. Interference of daunomycin (Da) with the stathmokinetic index in synchronized cultures. Daunomycin at 2 different concentrations was introduced for successive 1-hr periods (from the 3rd to the 9th hr after the end of the synchronization) together with VLB. The cultures were successively washed and incubated until the 9th hr in the medium containing the same concentration of VLB.
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Thereafter, the inhibiting effect of thymidine-$^3$H on DNA synthesis becomes less pronounced.

In order to determine the action of daunomycin on RNA synthesis during the S and G$_2$ phases, the cultures were treated at 1-hr intervals (from the end of synchronization until mitosis) and then immediately fixed (Chart 10). The treatment with the antibiotic (0.1 $\mu$g/ml) induces a strong inhibition of RNA synthesis, particularly in the nucleolus (see Chart 5).

Now the question arises of a possible relationship between the inhibition of RNA synthesis in the G$_2$ phase and the subsequent inhibition of mitotic activity. For clarification of this point, cultures were treated with different daunomycin concentrations for 1-hr periods from the 3rd to the 6th hr after the synchronization treatment. The effect on both the uridine-$^3$H incorporation in nuclear structures and the mitotic index was observed. In this experiment (Chart 11), the G$_2$ phase of RNA synthesis precedes by 2 hr the mitotic peak. There is notable reduction in uridine-$^3$H uptake during the peak period of RNA synthesis at daunomycin concentrations higher than 0.025 $\mu$g/ml. The mitotic activity, however, is lowered by a concentration of 0.0125 $\mu$g/ml when the antibiotic is added to the medium from the 6th to the 7th hr, i.e., in the period of maximum mitotic activity. Hence, the antimitotic effect does not necessarily seem to be related to the inhibiting effect on the RNA synthesis that occurs in G$_2$ phase.

DISCUSSION

The rate of RNA synthesis in rat fibroblast cultures

Chart 8. Interference of daunomycin (Da) at different concentrations with deoxycytidine-$^3$H incorporation. Batches of cultures were put into contact with the antibiotic and the labeled precursor for 1-hr periods and then immediately fixed. Left, mitotic behavior of control cultures; thick lines, time of antibiotic treatments.

Chart 9. Action of daunomycin (Da) on the incorporation of thymidine-$^3$H ($Thym-^3$H) and uridine-$^3$H ($Ur$) by synchronized cultures of rat fibroblasts. After the mitotic wave, the cells were treated with daunomycin (0.5 $\mu$g/ml) over 2-hr intervals. The cultures were put into contact with uridine-$^3$H during daunomycin treatment and immediately fixed. Another batch of cultures similarly treated was put into contact with thymidine-$^3$H from the 21st to the 29th hr and then fixed. Left, mitotic behavior of control cultures; thick lines, time of antibiotic treatments.
undergoes considerable variations during the different stages of the cell cycle. The uridine-$^3$H incorporation begins to increase 2 hr before the onset of the mitotic wave both in the nucleolus and in the extranucleolar area of the nucleus and reaches a maximum 1 or 2 hr before the peak of the mitotic wave. Considering the length of the G2 phase (2 hr), is is reasonable to conclude that RNA synthesis occurs in the nuclei when the DNA synthesis is already complete and the mitotic process has not yet begun. (Nucleolar RNA contributes mainly to this increase, while extranucleolar RNA plays a minor role.) Towards the end of G2, RNA synthesis progressively diminishes and remains at a very low level during mitosis and the first hours of the G1 phase. Then the RNA synthesis again begins to increase, and halfway through G1 a well-defined increase in incorporation of labeled uridine is observed. Once again, this is due mainly to the presence of nucleolar RNA. Taylor (23) and Robbins and Sharff (17) did not observe this periodical RNA synthesis in synchronized cultures. However, both these authors measured the rate of synthesis on samples of cells withdrawn from the cultures.

Another point which deserves consideration is the use, in our experiments, of diploid cells after the first in vitro transplant.

How far could the antimitotic effect of daunomycin be related to the inhibiting effect of RNA synthesis? One of the points of highest sensitivity to daunomycin, as far as the antimitotic activity is concerned, occurs in the G2 phase. Therefore, the inhibitory effect on the mitotic process obtained by treating the cells during this phase must be related to the interference with those metabolic events which occur when the DNA synthesis is already complete and before the mitotic process begins. The experiments carried out to determine the influence exerted by daunomycin on RNA synthesis during the G2 phase clearly demonstrate an inhibition of uridine-$^3$H incorporation during the hours immediately preceding mitosis. This inhibition, involving mainly the nucleolar structures and to a lesser extent the extranucleolar ones, is greatest when the rate of RNA synthesis reaches a maximum in the control cultures. It would therefore be reasonable to assume that the nuclear RNA synthesized during G2 plays an important role in the occurrence of mitosis. A more detailed analysis of the results shows that the relationship between the inhibiting effect on RNA synthesis during G2 and on mitosis is not a strict one. In fact, the occurrence of mitosis is inhibited by concentrations of daunomycin which have no effect on the synthesis of RNA; furthermore, mitosis can be inhibited by treating the cells after the period of maximum RNA synthesis has passed.

Thus, the mitotic inhibition achieved under these conditions cannot be ascribed to a block in the synthesis of RNA in the G2 period and other metabolic events must come into play to explain the antimitotic effect of daunomycin. This does not mean that this RNA is not important for triggering off the mitotic process. In fact, doses of daunomycin which are able to inhibit this RNA synthesis are also able to prevent the cells from entering the mitotic stage. Modification of the structure of daunomycin which impairs the binding capacity to DNA is also very effective in reducing the antimitotic activity of this drug. Both are, in fact, considerably reduced in the N-acetyl daunomycin derivative (9). There is also the possibility that the antimitotic effect observed under these conditions may be due to a modification of the tertiary structure of DNA, which does not permit its passage from the interphase to the mitotic chromosomes. Experiments carried out to determine the importance of the RNA synthesized during G1 on subsequent synthesis demonstrated that daunomycin causes a nearly complete inhibition of the nucleolar uridine-$^3$H incorporation and a considerable but less evident reduction of uridine-$^3$H incorporation into the extranucleolar region of the nucleus. The maximum inhibition of DNA synthesis during the following S phase is reached when the cells are treated in the middle of the G1 phase, i.e., when maximum RNA synthesis occurs in control cells. These observations are consistent with the hypothesis that in the middle stages of
the G₁ phase a sequence of biochemical events essential to DNA replication occurs in the cell (11). As these events can be influenced by an inhibition of DNA-dependent RNA synthesis, they could be related to the formation of RNA molecules bearing the information necessary for the synthesis of the enzymes essential to DNA replication.

The pattern of DNA synthesis shows marked variations during the S phase. The autoradiographic determinations of nuclear labeling show 2 distinct peaks of DNA synthesis localized, respectively, in the early and late S phases. The 2nd wave of DNA synthesis, according to postulates by another worker (22), may be reasonably identified as the replication of heterochromatin. The inhibitory effect of daunomycin on DNA synthesis varies according to the concentration of the drug and the period of the S phase during which the cells come in contact with daunomycin. DNA synthesis in the early S phase is not inhibited and even increases with low doses of daunomycin, as shown by the increase in deoxycytidine-³H incorporation. This increased incorporation must be interpreted as a lack of DNA synthesis regulation, rather than a real increase.

On the contrary, inhibition of deoxycytidine-³H incorporation proportional to the antibiotic concentration is observed when the cells are treated in the last stages of the S phase. It is in this period that the maximum binding of daunomycin to the nuclear structures has been observed (20). Since synthesis of heterochromatin occurs during the last stages of the S phase, it is probable, as already noted for actinomycin (22), that the maximum binding of daunomycin also occurs during the synthesis of heterochromatin and that this impairs the synthesis of DNA.

In conclusion, the uptake of daunomycin produces different effects according to the physiological activity of the cells at the time of treatment. In cells in the G₁ phase, there is inhibition of synthesis of enzymes relevant to DNA replication. In cells in the S phase, there is inhibition of DNA replication, not necessarily followed by mitotic inhibition; irreversible damage to the chromosomal structures appears in a high percentage of the cells. In cells in the G₂ phase, there is blockage of the synthesis of nuclear and particularly nucleolar RNA, which could be essential for triggering off the mitotic process. In cells in mitosis, there is mitotic blockage when the drug is given just a few minutes before the prophase.

All these effects are related to the ability of daunomycin to bind to DNA, and can be obliterated or reduced by modifying the daunomycin molecule in such a way that its binding capacity with DNA is reduced. In fact, the N-acetyl derivative of daunomycin, endowed with a low binding capacity to DNA (4), shows almost none of these effects.

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