INHIBITING EFFECT OF THE NEW CYTOTOXIC ANTIBIOTIC DAUNOMYCIN ON NUCLEIC ACIDS AND MITOTIC ACTIVITY OF HELA CELLS

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ABSTRACT

The effect has been studied of Actinomycin D, Daunomycin (Da.), and Da. N acetyl derivative on mitotic activity and on the nucleic acid synthesis of *in vitro* HeLa cell cultures. The experiments were carried out by means of the radioautographic technique using stripping films. The relative uptake of thymidine-H^3 and uridine-H^3 was determined by means of the reduced silver grain count present in the nuclei of controls and treated cells. The mitotic activity and thymidine incorporation were noticeably reduced by Daunomycin and Actinomycin, whereas both processes appeared less affected by Da. N acetyl derivative. As regards nuclear RNA synthesis, all three antibiotics at low doses chiefly inhibit nucleolar RNA synthesis. On the other hand, whilst Actinomycin at higher doses causes an almost total inhibition of the synthesis of the whole nuclear RNA, in Daunomycin- and Da. N acetyl derivative–treated cells extranucleolar RNA synthesis is less susceptible to inhibition.

Daunomycin (Da.) is a new antibiotic isolated from cultures of a *Streptomyces* named *S. peucetius* (5, 8). A product with the same physicochemical properties has also been isolated by Dubost *et al.* (7). This substance is a glucoside (hydrochloride $C_{27}H_{29}NO_{10}\cdot HCl$) and by acid hydrolysis yields a pigmented aglycone (Daunomycinone) (Fig. 1) as well as a new amino sugar (Daunosamine) (1, 2).

The physicochemical changes of a solution of Daunomycin in the presence of DNA and DNA itself suggest a specific binding of the antibiotic to DNA (3). On tissue culture of animal cells, the most evident morphological effects (4) are 1) early nucleolar changes such as swelling and fragmentation; 2) chromosomal aberrations; and 3) preprophasic mitotic blockage.

In preliminary radioautographic experiments, an interference of the antibiotic with uridine-H^3 incorporation into nuclear RNA and with thymidine-H^3 into DNA was observed (15). In view of the similarity of the above mentioned results with those reported for Actinomycin D (13), a comparative investigation of the activity of the two antibiotics and of a derivative of Da. that has a reduced capacity for binding to DNA was considered advisable.

MATERIALS AND METHODS

The experiment was carried out on HeLa cells grown in a medium consisting of lactalbumin hydrolysate at 0.5 per cent Hanks' salt solution and decomplementized calf serum at 5 per cent.

In the first experiment, Actinomycin D (0.1, 0.5 and 1 μg/ml)[1], Daunomycin (0.1, 0.2, 0.5, and 1 μg/ml)[2], and Da. N acetyl derivative[3] (5, 10, 20, and

[1] Merck, Sharp, and Dohme Co., West Point, Pennsylvania.

[2, 3] Farmitalia, Milan, Italy.

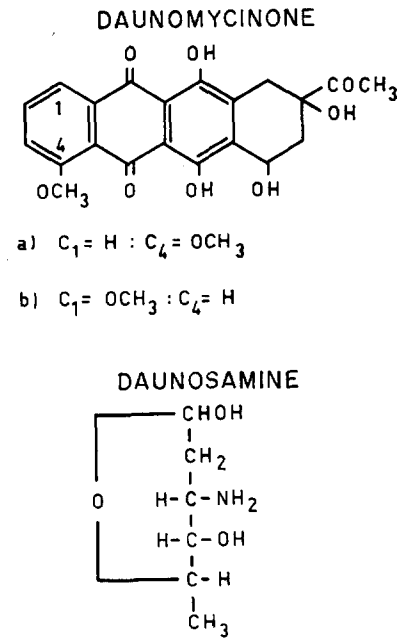

FIGURE 1 Chemical structure of Daunomycin.

40 μg/ml) were added 48 hours after culture transplantation, at the same time that the change of medium took place.

Thymidine-H^3 (specific activity 160 mc/mM)[4] at the dose of 2.5 μc/ml was added to the medium 1 hour after the beginning of the treatment. Sample cells were withdrawn at different intervals, fixed in Bouin's, washed in TCA at 5 per cent and 4°C as well as in carrier thymidine.

In a subsequent experiment, Actinomycin D (0.1 μg/ml), Daunomycin (0.1 and 1 μg/ml), and Da. N acetyl derivative (5 to 10 μg/ml) were added to the medium 48 hours after transplantation. After 45 minutes' treatment, uridine-H^3 (1.2 c/mM)[5] at the dose of 2 μc/ml was added to the medium for 30 minutes. One group of cells was treated with ribonuclease. The enzymatic digestion was effected with ribonuclease[6], 0.2 mg/ml in 0.02 M sodium phosphate buffer solution (McIlvaine) at a pH of 7.0 for 90 minutes at 55°C. The slides were covered with AR 10 Kodak stripping film (11) and developed after an exposure ranging from 2 to 4 days. The cells were stained through the emulsion with Mayer's hematoxylin.

The relative uptake of thymidine-H^3 and uridine-H^3 was determined at each interval by means of a grain count of groups of 60 cells.

[4] Radiochemical Centre, Amersham, England.
[5] Radiochemical Centre, Amersham, England.
[6] Sigma Chemical Company, St. Louis, Missouri.

RESULTS

1. Effect on Mitotic Activity and Thymidine-H^3 Incorporation into DNA

ACTINOMYCIN D: The mitotic activity of the treated cells is similar in all three tested doses (Fig. 2 *a*). In the first 4 hours, the mitotic index values show a slight decrease. A more consistent reduction of mitosis is observed only after the 4th hour. After a 6-hour treatment with 1 μg/ml of Actinomycin D, an increase in the number of metaphase cells is observed and the cells appear severely damaged, whilst 8 hours later they have completely degenerated. It seems probable that, at high antibiotic concentrations, in addition to the preprophasic poisoning generally caused by Actinomycin D, an impairment to the accomplishment of mitosis is observed. Thymidine-H^3 incorporation into the treated cells was soon reduced, in proportion to Actinomycin D concentration. Six hours after the administration of Actinomycin D, no further thymidine incorporation into the nuclei was evident (Fig. 2 *b*).

DAUNOMYCIN: Da. causes an immediate and increasing reduction in the mitotic index (Fig. 3 *a*). Four hours after a dose of 1 μg and 6 hours after doses of 0.1, 0.2, and 0.5 μg, mitosis has practically disappeared. The incorporation of thymidine appears remarkably reduced 2 hours after the beginning of the treatment (Fig. 3 *b*). DNA synthesis decreases in proportion to the dose used but, contrary to what has been observed in the Actinomycin D-treated cells, it continues for about 8 hours.

DA. N ACETYL DERIVATIVE: The mitotic index appears completely unaffected at the doses of 5 and 10 μg (Fig. 4 *a*). At 20 μg, the mitotic activity is partially inhibited in the first 2 hours but then rises. The dose of 40 μg causes an increasing reduction in the mitotic activity, with a behavior very similar to that of Daunomycin: a prevalence of metaphasic stages with chromosomal breaks is observed. Thymidine-H^3 incorporation is not affected by doses of 5 and 10 μg, which cause marked alterations in the nucleolus, at least in the first 8 hours of treatment (Fig. 4 *b*).

Cultures treated with 40 μg of Da. N acetyl derivative for 24 hours show a lower percentage of labeled cells compared with the controls (Fig. 5). This decrease may be related to the preprophasic blockage caused by the antibiotic; in fact, in the treated cultures the mitotic index is reduced

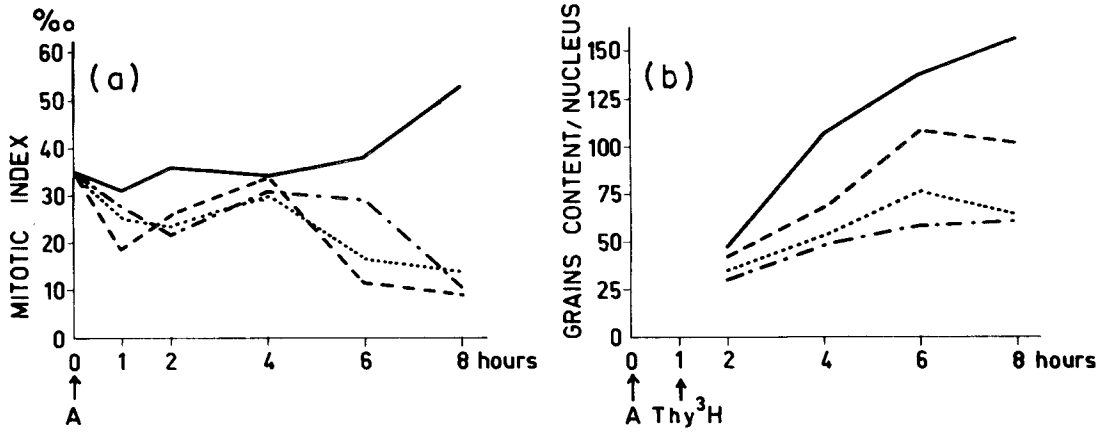

FIGURE 2 *a* Variation of mitotic index after treatment with different doses of Actinomycin D.

FIGURE 2 *b* Thymidine H^3 incorporation into control cells and Actinomycin D–treated cells.
Control ———; 0.1 μg/ml --------; 0.5 μg/ml ·····; 1 μg/ml –·–·–.

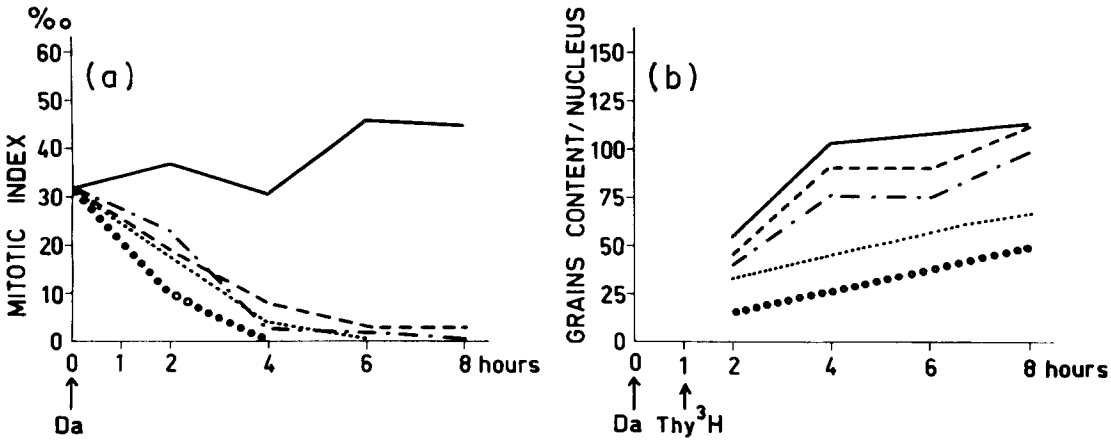

FIGURE 3 *a* Variation of mitotic index after treatment with different doses of Daunomycin.

FIGURE 3 *b* Thymidine H^3 incorporation into control cells and Daunomycin-treated cells.
Control ———; 0.1 μg/ml --------; 0.2 μg/ml –·–·–; 0.5 μg/ml ·····; 1 μg/ml ••••••.

to 70 per cent, whereas in the control cultures the division of labeled elements proceeds normally. At the same time, a greater thymidine-H^3 incorporation per nucleus is observed in treated cultures as compared with the controls (Fig. 5). In the treated cultures, histograms show a larger number of highly labeled nuclei, and nuclei with a grain content higher than the maximum value found in the controls (Fig. 6).

It is, therefore, evident that even with high dosage of Da. N acetyl derivative, sufficient to stop the mitotic activity, the DNA synthesis can proceed.

2. *Effects on Uridine-H^3 Incorporation into RNA*

The radioactivity present in the cells after uridine-H^3 contact is completely removed by RNase digestion. Uridine-H^3 uptake was determined over the nucleolus as well as in the extranucleolar zone of the interphasic nuclei.

The grain count, carried out in the different cellular structures, shows (Fig. 7) that Actinomycin D at the dose of 0.1 μg/ml brings about a marked reduction in nucleolar RNA synthesis (75 per cent) and a lower inhibition of the RNA that is

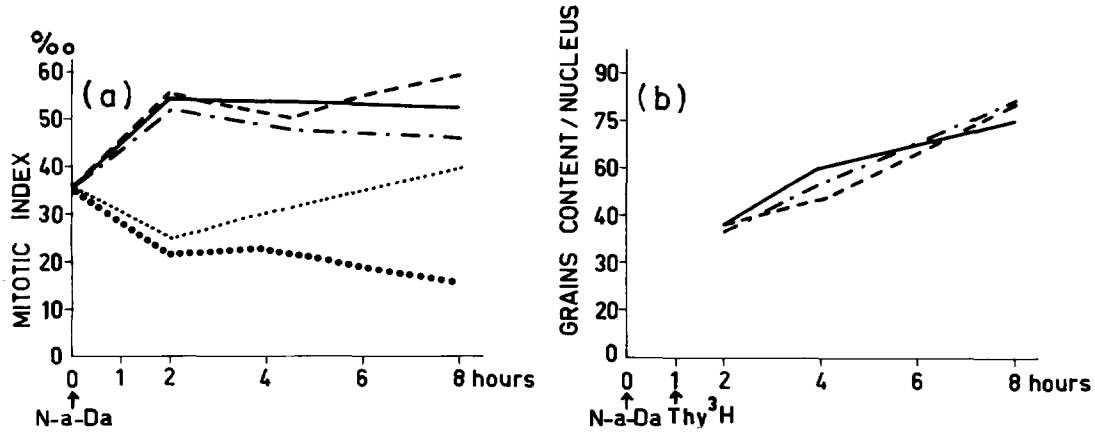

FIGURE 4 *a* Variation of mitotic index after treatment with different doses of Da. N acetyl derivative.

FIGURE 4 *b* Thymidine H^3 incorporation into control cells and Da. N acetyl derivative-treated cells. Control ———; 5 μg/ml ---------; 10 μg/ml –·–·–; 20 μg/ml ·····; 40 μg/ml ••••••.

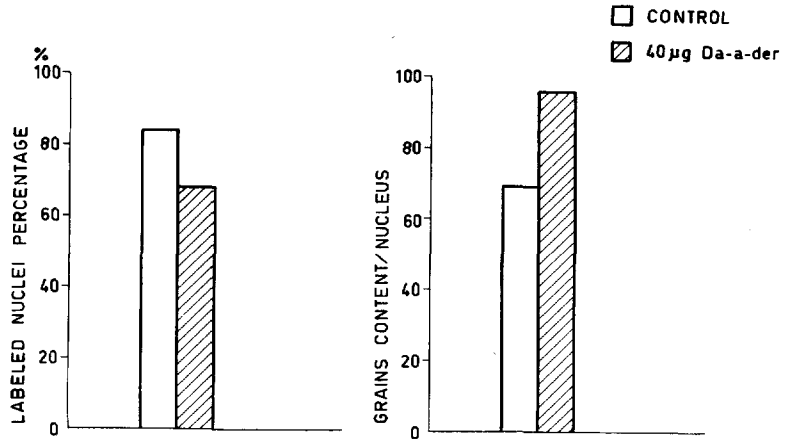

FIGURE 5 Thymidine H^3 incorporation into control cultures and cultures treated for 24 hours with 40 μg/ml of Da. N acetyl derivative.

synthesized in the extranucleolar zone of the nucleus (30 per cent). At a dose ten times higher, nuclear RNA synthesis is completely inhibited, and, in the extranucleolar zone of the nucleus, too, uridine-H^3 incorporation is reduced by 95 per cent.

After treatment with Daunomycin (0.1 μg/ml), a result similar to that found in Actinomycin D–treated cells is observed; *i.e.* RNA synthesis is inhibited diversely at the level of the two nuclear structures; that is to say, there occur a 70 per cent reduction in RNA synthesized in the nucleolus and only a 15 per cent reduction in RNA synthesized in the extranucleolar zone of the nucleus. Conversely to what has been observed in Actinomycin D–treated cells, even by increasing the dose of the antibiotic 10 times the degree of inhibition remains constant.

Da. N acetyl derivative is active only at a much higher dose, as compared with the previously mentioned antibiotics. In fact, 50 times higher doses of Da. N acetyl derivative are required to reach the same degree of inhibition as that obtained with 0.1 μg/ml of Daunomycin. Similar to what has been found with regard to Daunomycin, the degree of inhibition of nucleolar RNA and extranucleolar RNA remains constant even when the concentration of the antibiotic in the medium is increased.

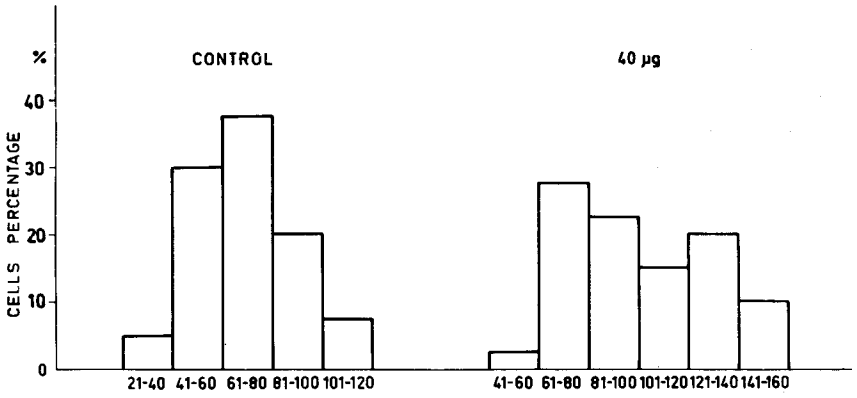

FIGURE 6 Frequency of cells showing different grain contents in control cultures and in cultures treated for 24 hours with 40 μg/ml of Da. N acetyl derivative.

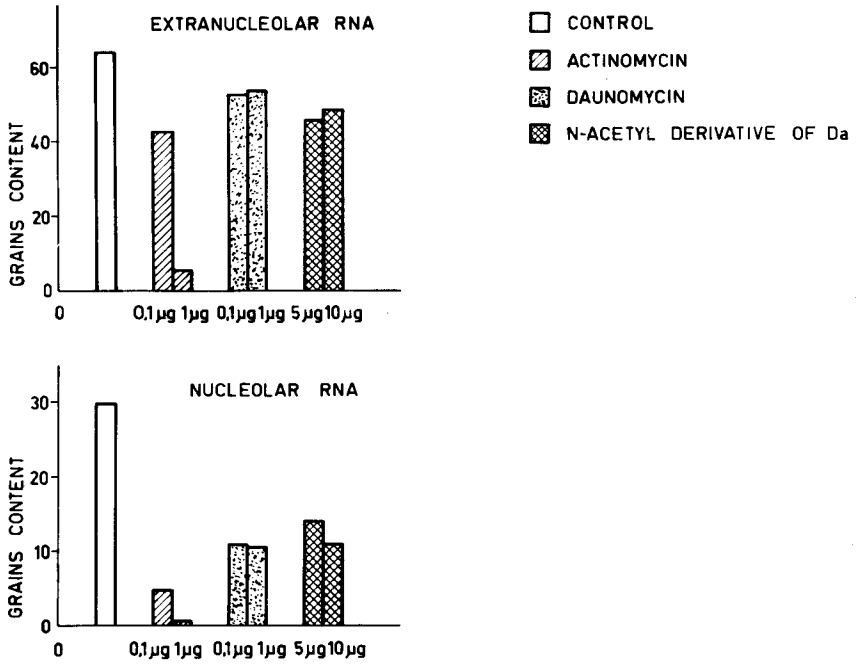

FIGURE 7 Uridine H^3 incorporation into extranucleolar RNA and nucleolar RNA in control cells and in cells treated with different antibiotics after a 45-minute treatment and a 30-minute contact with the precursor.

DISCUSSION

The inhibiting effect of Daunomycin on thymidine-H^3 incorporation into DNA of *in vitro*-cultured HeLa cells accords with the action exerted by these substances on the activity of DNA polymerase (10). The close affinity of Da. to DNA indicates that, as in the case of Actinomycin (9, 12), the effect might be related to the binding of the dye to DNA and not to a direct effect on the enzyme. This assumption is supported by the observation that Da. N acetyl derivative, which has a diminished capacity to bind to DNA (3), also has a reduced capacity to inhibit the incorporation of different precursors into DNA and RNA. In Actinomycin D–treated cells there is a chronological relationship between inhibition

of mitosis and inhibition of DNA synthesis. The inhibition of mitotic activity exerted by Da. cannot be related only to the inhibition of DNA synthesis. In fact:

1. The reduction of the mitotic index appears 1 hour after the beginning of the treatment; that is to say, cells which have already completed the DNA synthesis and are in G_2 phase or in mitosis are also affected.

2. A notable inhibition of the mitotic index may also take place with doses of Da. which have no action or only slight action on DNA synthesis.

3. In cultures of HeLa cells treated with an amount of Da. N acetyl derivative sufficient to reduce considerably the mitotic activity, the DNA synthesis can proceed.

It may also be excluded that the antimitotic effect is correlated to a metabolic effect on RNA synthesis. The dissociation of the two phenomena is in fact evident in cells treated with low doses of Da. N acetyl derivative (5 and 10 μg/ml) in which a marked inhibition of RNA synthesis is observed (6), whilst the mitotic activity is not affected.

It is, therefore, possible that the blockage of mitosis and the serious chromosomal damage caused by Da. are due to the physicochemical changes in DNA structures following linkage with the antibiotic. In this respect, the effect of Daunomycin may usefully be compared to that of proflavine, a substance which also shows a specific binding with DNA (14).

As far as the effect exerted on RNA synthesis is concerned, our radioautographic observations show that, whereas at lower doses of Actinomycin D the inhibition of uridine-H^3 incorporation is higher in the nucleolus than in the extranucleolar zone, higher doses bring about a practically complete inhibition of the synthesis of the whole nuclear RNA. In Daunomycin- and Da. N acetyl derivative–treated cells, extranucleolar RNA synthesis takes place also at higher tested doses.

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