Relationship between Nucleolar Microspherule Formation by Urethan and Inhibition of RNA and Protein Synthesis

Luciano Lombardi
Division of Experimental Oncology A, Istituto Nazionale per lo Studio e la Cura dei Tumori, Via G. Venezian 1, 20133 Milan, Italy

SUMMARY

Liver tissues of suckling C3Hf mice treated with urethan, a carcinogen and inhibitor of RNA synthesis, and with cycloheximide, an inhibitor of protein synthesis, were studied by electron microscopy and by light microscope radioautography.

At 4 and 12 hr after treatment with a single dose of urethan (1 mg/g body weight), [3H]uridine incorporation in hepatocyte nuclei was 38 and 9% lower than in controls and became 32% higher at 24 hr. Nucleolar microspherules were found 1.5 and 4 hr after the treatment, increased considerably in number at 10 and 12 hr, and decreased at 24 hr. These results suggest an inverse relationship between RNA synthesis and formation of microspherules.

Cycloheximide alone (3 mg/kg body weight) produced microspherules, whereas in combination with a dose of 1 or 2 mg of urethan per g body weight it partially prevented or potentiated, respectively, the microsphere production.

INTRODUCTION

Very little is known of the mode of action of urethan as a carcinogen; however, there is considerable evidence that the binding of this substance and of chemical carcinogens generally to the target cell DNA is an essential step in carcinogenesis (12).

Urethan, or a metabolite, binds covalently to the liver cell DNA (1, 22), and this biochemical reaction seems to depend on the proliferative state of hepatocytes since partial hepatectomy increases the binding of urethan to liver DNA in adult rats (23) and mice (21). Like other substances that bind to DNA, urethan has been shown to affect the metabolism of nucleic acids (5-7). RNA synthesis was inhibited by this substance in newborn mice (5) and hepatectomized mice. Such microspherules consisted primarily of proteins, and we suggested that the nature of these proteins is ribosomal (17). A slowing down of RNA synthesis by urethan could produce clumps (microspherules) of unused ribosomal proteins.

To verify this working hypothesis, in the present research we studied in parallel nucleolar microsphere number and [3H]uridine incorporation into rRNA in hepatocytes of 7-day-old C3Hf mice treated with a carcinogenic dose of urethan. In addition we investigated whether a previous treatment with an inhibitor of protein synthesis, cycloheximide, could interfere with the formation of microspherules produced by urethan.

MATERIALS AND METHODS

Animals. Seven-day-old C3Hf mice were used. All injections were administered i.p. Treatments. Three groups of 8 to 11 mice were given injections of 1 mg of urethan per g of body weight as a 6% solution in sterile distilled water, and 3, 11, or 23 hr later the mice were treated with 40 μCi of [5-3H]uridine (specific activity, 27 mCi/mmol) per g of body weight. A control group of 9 untreated mice was given [5-3H]uridine only. All the animals were killed 1 hr after uridine.

Other groups of 8 to 12 animals were treated either with a single dose of 1 or 2 mg/g body weight of urethan as a 6 or 12% solution, respectively; with a dose of cycloheximide, 3 mg/kg body weight, in a concentration of 0.03% in sterile distilled water; or with both drugs. The mice were killed and observed as indicated in Table 2. A control group consisted of 14 untreated mice.

Electron Microscopy. Hepatic tissues were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1.5 hr at 4°C and postfixed in 1% osmium tetroxide in Millonig’s buffer for 1 hr at 4°C, dehydrated in alcohol, and embedded in Epon 812.

For each animal ultrathin sections cut from 3 different tissue blocks and stained with uranyl acetate and lead citrate were observed. Fibrillar aggregates and microspherules found in all hepatocyte nucleoli of 1 section per block were counted. From 414 to 717 nucleoli per experimental group were studied. The counting was expressed as number of fibrillar aggregates plus microspherules per nucleolus. The significance of the data was checked by Fisher’s F test.

A size estimation of microspherules and fibrillar aggregates was performed. The negatives of nucleoli photographs, all taken at the same magnification of ×14,000, were enlarged 5 times. The microsphere and fibrillar aggregate outlines were drawn on paper sheets, and their diameter was measured.

For enzyme digestion ultrathin sections were treated with a 10% solution of 12 volumes of hydrogen peroxide for 1 hr.
at room temperature and then digested for 2 hr at room temperature with a 0.5% Pronase solution in distilled water at pH 7.4 (17). The sections were then stained with uranyl acetate and lead citrate.

**Light Microscope Radioautography.** A tissue fragment was dissected from each of the 4 lobes of liver. The fragments were fixed with 4% neutral formaldehyde for 24 hr at 4°C, then washed for 2 days in several changes of ice-cold M/15 Sorensen's phosphate buffer, pH 7.3, to remove the acid-soluble RNA precursors (18). After the fragments were washed, they were routinely dehydrated and embedded in paraffin.

Paraffin sections (5 μm) were mounted on glass slides, coated with NTB3 Kodak liquid emulsion, and exposed at 4°C for 1 week. The radioautographs were developed in D 170 for 2 min at 18°C and fixed for 2 min in Kodafix (13). After a rinsing in tap water, the slides were stained with Harris' hematoxylin and alcoholic eosin and mounted with Canada balsam.

Radioautographic quantitation was carried on 3 sections for each liver fragment. Silver grains were counted over the entire cut surface of 10 hepatocyte nuclei/section; 120 nuclei/animal were observed. The area of each nucleus was measured using a micrometer inserted into a ×10 ocular of a microscope fitted with a ×100 objective, each micrometer square thus including a 4-sq μm area. The grain counts were expressed as number of silver grains per unit area of 4 sq μm. The values were corrected for background and their significance was checked by Fisher’s F test.

**RESULTS**

**Microspherule Formation and [3H]Uridine Incorporation after Treatment with Urethan Alone.** Confirming our previous observations (16, 17), aggregates of dense fibrillar material were observed within the meshes of the nucleolonema of hepatocyte nuclei of control mice (Fig. 1). At 4 hr after urethan treatment, microspherules were evident, several being denser than fibrillar aggregates, although in some cases the difference of density was so small that it was hard to distinguish between the 2 structures. As shown in Table 1, the mean total number of microspherules plus fibrillar aggregates per nucleolus observed at 4 hr was not significantly higher (11%) than was the mean number of fibrillar aggregates of control group. A considerable increase of total number of microspherules plus fibrillar aggregates (76% higher than in the control) was found at 12 hr, largely due to a great number of well-defined microspherules (Fig. 2). At 24 hr there were fewer microspherules, and hence the total number of microspherules plus fibrillar aggregates was 35% over the control.

In Table 1 are reported also the radioautographic grain counts, which did not show large dispersion within each experimental group. The mean values of the 3 urethan-treated groups were all significantly different from that of the control group. The grain count was 38% under the control value at 4 hr, only 9% under at 12 hr, and augmented to 32% over the controls 24 hr after urethan.

**Microspherule Formation after Cycloheximide plus Urethan Treatment.** The mean numbers of microspherules and fibrillar aggregates observed in the nucleoli of the different animals within each experimental group varied significantly as shown by the intragroup variance analysis. A similar fluctuation was observed in the control group and in the treated groups, and no large dispersion was noted in the pool of countings for each group (Table 2).

Cycloheximide alone produced the formation of typical microspherules (Fig. 3). Such effect was rapidly reversible. In fact, as shown in Table 2, 2.5 and 11 hr after the treatment the mean total number of microspherules plus fibrillar aggregates was, respectively, 70% higher (p < 0.01) and 7% lower (not significant) than the mean number of fibrillar aggregates of the control group.

Microspherules were found as soon as 1.5 hr after the treatment with the lower dose of urethan, their number, fibrillar aggregates included, being only 5% higher (not significant) than the number of control fibrillar aggregates. At 10 hr after treatment, microspherule number was considerably increased and thus the total number of microspherules and fibrillar aggregates was 65% higher (p < 0.01) than in the control.

The double treatment by cycloheximide followed by the lower dose of urethan induced different effects according to the time of injection of both substances. In the group that was given urethan 1 hr after cycloheximide and observed 1.5 hr afterward, the number of microspherules

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**Table 1**

<table>
<thead>
<tr>
<th>Time after urethan treatment (hr)</th>
<th>No. of mice</th>
<th>Fibular aggregates and microspherules</th>
<th>Radioautographic grains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>% control</td>
<td>F</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>0.54 ± 0.04* (414)</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>0.60 ± 0.04 (415)</td>
<td>111</td>
</tr>
<tr>
<td>12</td>
<td>11</td>
<td>0.95 ± 0.05 (458)</td>
<td>176</td>
</tr>
<tr>
<td>24</td>
<td>8</td>
<td>0.73 ± 0.04 (416)</td>
<td>135</td>
</tr>
</tbody>
</table>

* The significance of values was checked by Fisher’s F test by comparing the control value with those of each treated group.

* Mean ± S.E. per hepatocyte nucleolus.

* Numbers in parentheses, number of observed nucleoli.

* Mean count ± S.E. per 4 sq-μm unit area of 120 nuclei/animal.

* NS, not significant.
Table 2

Count and size estimation of fibrillar aggregates and microspherules in hepatocyte nucleoli of mice treated with cycloheximide and urethan

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Count</th>
<th>% control</th>
<th>F</th>
<th>( p^b )</th>
<th>Diameter (nm)</th>
<th>% control</th>
<th>F</th>
<th>( p^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14</td>
<td>0.40 ± 0.03 (717)</td>
<td>100</td>
<td>76 ± 1.4* (77)</td>
<td>100</td>
<td>76 ± 1.4* (77)</td>
<td>100</td>
<td>76 ± 1.4* (77)</td>
<td>100</td>
</tr>
<tr>
<td>Cycloheximide (2.5 hr)</td>
<td>12</td>
<td>0.68 ± 0.05 (522)</td>
<td>170</td>
<td>84 ± 1.3 (118)</td>
<td>111</td>
<td>84 ± 1.3 (118)</td>
<td>111</td>
<td>84 ± 1.3 (118)</td>
<td>111</td>
</tr>
<tr>
<td>Urethan, 1 mg (1.5 hr)</td>
<td>8</td>
<td>0.42 ± 0.04 (470)</td>
<td>105</td>
<td>76 ± 2.1 (70)</td>
<td>100</td>
<td>76 ± 2.1 (70)</td>
<td>100</td>
<td>76 ± 2.1 (70)</td>
<td>100</td>
</tr>
<tr>
<td>Cycloheximide + urethan, 1 mg (1.5 hr)</td>
<td>8</td>
<td>0.84 ± 0.05 (434)</td>
<td>210</td>
<td>96 ± 1.3 (90)</td>
<td>127</td>
<td>96 ± 1.3 (90)</td>
<td>127</td>
<td>96 ± 1.3 (90)</td>
<td>127</td>
</tr>
<tr>
<td>Cycloheximide (11 hr)</td>
<td>8</td>
<td>0.37 ± 0.03 (558)</td>
<td>93</td>
<td>99 ± 2 (54)</td>
<td>131</td>
<td>99 ± 2 (54)</td>
<td>131</td>
<td>99 ± 2 (54)</td>
<td>131</td>
</tr>
<tr>
<td>Urethan 1 mg (10 hr)</td>
<td>9</td>
<td>0.66 ± 0.04 (471)</td>
<td>165</td>
<td>74 ± 1.8 (76)</td>
<td>98</td>
<td>74 ± 1.8 (76)</td>
<td>98</td>
<td>74 ± 1.8 (76)</td>
<td>98</td>
</tr>
<tr>
<td>Cycloheximide + urethan, 1 mg (10 hr)</td>
<td>8</td>
<td>0.45 ± 0.02 (425)</td>
<td>113</td>
<td>102 ± 2.6 (55)</td>
<td>135</td>
<td>102 ± 2.6 (55)</td>
<td>135</td>
<td>102 ± 2.6 (55)</td>
<td>135</td>
</tr>
<tr>
<td>Urethan, 2 mg (1.5 hr)</td>
<td>12</td>
<td>0.65 ± 0.04 (578)</td>
<td>163</td>
<td>79 ± 1.1 (104)</td>
<td>104</td>
<td>79 ± 1.1 (104)</td>
<td>104</td>
<td>79 ± 1.1 (104)</td>
<td>104</td>
</tr>
<tr>
<td>Cycloheximide + urethan, 2 mg (1.5 hr)</td>
<td>8</td>
<td>1.80 ± 0.03 (436)</td>
<td>450</td>
<td>86 ± 0.8 (245)</td>
<td>113</td>
<td>86 ± 0.8 (245)</td>
<td>113</td>
<td>86 ± 0.8 (245)</td>
<td>113</td>
</tr>
</tbody>
</table>

\( a \) The interval between the 2 treatments was 1 hr. Cycloheximide was given at 3 mg/g body weight. The indicated dose of urethan was per g body weight.

\( b \) The significance of values was checked by Fisher's \( F \) test by comparing the control value with those of each treated group.

\( ^* \) Mean ± S.E. per hepatocyte nucleolus.

\( ^\dagger \) Numbers in parentheses, number of observed nucleoli.

\( ^\ddagger \) Numbers in parentheses, number of fibrillar aggregates and microspherules measured.

\( ^\dagger\dagger \) Numbers in parentheses, interval between the last treatment and sacrifice.

\( ^\ddagger\ddagger \) NS, not significant.

A variance analysis by Fisher's \( F \) test performed by comparing the microspherule plus fibrillar aggregate counts of the groups treated by urethan plus cycloheximide with the counts of the corresponding groups treated by urethan or cycloheximide only showed that the differences among the 3 experimental groups considered together were significant (\( p < 0.01 \)). The differences between the 3 groups treated by urethan only versus the 2 groups treated by cycloheximide alone and the 3 groups given cycloheximide plus urethan were also significant (\( p < 0.01 \)). The differences between the 3 groups treated by cycloheximide only versus the 3 groups with both treatments for the lower dose of urethan were less significant (\( p < 0.05 \)) at the earlier observation and not significant at the later observation, whereas for the higher dose of urethan the significance increased to \( p < 0.01 \).

The values obtained by measuring the diameter of fibrillar aggregates and microspherules are reported in Table 2. After urethan alone, their mean size was almost identical to that of the control fibrillar aggregates, whereas the 2 groups treated with cycloheximide had microspherules 11 and 31% larger, respectively, than did controls (\( p < 0.01 \)). The double treatment resulted in a slight further increase of the mean values, although some very large and dense microspherules were observed (Fig. 5). Samples of ultrathin sections from all control and experimen-
Mechanism of Nucleolar Microsphere Formation

Mental groups were observed after Pronase treatment. Both fibrillar aggregates and microspherules were entirely digested (Fig. 6).

DISCUSSION

Chemical substances that affect DNA-dependent RNA synthesis (25), nucleotide pool (25), and cleavage of 45S rRNA precursor (10) produce nucleolar alterations such as segregation, fragmentation, and degranulation. The inhibitors of protein synthesis that secondarily impair RNA synthesis have been reported (3, 8, 27) not to induce similar alterations except when administered at high doses (9).

The origin of another type of nucleolar lesion, the microspherules, is less known. However, a general agreement has been reached in ascribing their genesis to an alteration in rRNA synthesis (26). According to the report of Smetana and Busch (26), the occurrence of microspherules in untreated cells also denotes cellular pathology. Nevertheless, aggregates of dense fibrillar material similar to microspherules have been observed by Le Beux (15) in secretory neurones of rats and by us in the liver of untreated suckling mice (16). Recher et al. (24) reported that nucleolar microspherules consisted primarily of basic proteins, but according to other authors the presence of nucleic acids in these structures cannot be excluded (17, 28).

The data of this report confirm the relationship between inhibition of RNA synthesis by urethan and microsphere production and show that an inhibitor of protein synthesis, cycloheximide, administered at low doses, can also induce these nucleolar lesions.

The variations in total number of microspherules plus fibrillar aggregates in each experimental group seem to support our hypothesis (17) that both structures are due to accumulation of ribosomal proteins. Inhibition of RNA synthesis could produce an accumulation of unused ribosomal proteins and hence an increase of microsphere number. At 4 hr after urethan treatment, the appearance of microspherules coincided with the lowest uridine incorporation. The total number of microspherules plus fibrillar aggregates being similar to the number of fibrillar aggregates of the controls since early microspherules are probably formed by transformation of preexisting aggregates. The increased total number observed at 12 hr when radioactive reaction was still low must be due to a de novo formation of microspherules. At 24 hr, the recovery of nucleic acid metabolism, testified to by silver grain counts significantly higher than in the controls for some overcompensation phenomenon, resulted in a picture toward normalization of nucleolar structure.

According to these results, a specific inhibition of protein synthesis would prevent microsphere formation. We used cycloheximide; its specificity as an inhibitor of protein synthesis has been amply documented (4), with its differential effect on protein or RNA synthesis depending on the dose and on the proliferative state of target cells.

We selected a dose that should inhibit specifically protein synthesis (4). However, our data on the interaction between cycloheximide and urethan were confusing because cycloheximide alone produced more and larger microspherules than urethan alone. Perhaps this was due to an impairment of both protein and RNA synthesis, as found when cycloheximide was given to partially hepatectomized adult rats (11) or to rapidly growing cells in tissue culture (3). In our case, the failure to obtain a selective effect on protein synthesis could be due to the rapid growth of hepatic tissue in suckling mice or to a low rate of drug metabolism shown at this age (2).

It is well known that the ribosomes are assembled in the nucleolus through a multistep process in which the protein and RNA metabolisms are reciprocally related (19). The integrity of nucleolar structure is function dependent, and thus the inhibitors of both protein and RNA synthesis can produce nucleolar lesions. Cycloheximide probably induces microspherules by affecting the metabolism of the small fraction of preribosomal protein pool that has a rapid turnover and regulatory functions (14, 20). If urethan and cycloheximide produce microspherules by 2 different mechanisms, the administration of both substances would be expected to result in a summation effect. On the contrary, we noted some interference between the 2 substances when urethan was used at the lower level, whereas a potentiation effect was observed with the higher dose of urethan which induced nucleolar degranulation as well. Further investigation may clarify the mechanisms involved and also the nature of the lesions produced by the 2 drugs.

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Fig. 1. Nucleolus of a control mouse hepatocyte. Aggregates of dense fibrillar material are observed (arrow) within the meshes of the nucleolar network. Uranyl acetate and lead citrate, × 29,000.

Fig. 2. Hepatocyte nucleolus of a mouse killed 12 hr after the treatment with urethan (1 mg/g body weight). Numerous microspherules are found. Uranyl acetate and lead citrate, × 29,000.

Fig. 3. Hepatocyte nucleolus from a mouse killed 2.5 hr after cycloheximide treatment. Note 3 large microspherules. Uranyl acetate and lead citrate, × 29,000.

Fig. 4. Hepatocyte degranulated nucleolus of a mouse treated with urethan (2 mg/g body weight). Vacuoles are present in the remaining fibrillar material. Uranyl acetate and lead citrate, × 29,000.

Fig. 5. Hepatocyte nucleolus of a mouse treated with cycloheximide followed by urethan (2 mg/g body weight) and killed 1.5 hr after the last injection. Large microspherules are surrounded by a crown of granules. Uranyl acetate and lead citrate, × 29,000.

Fig. 6. Hepatocyte nucleolus of a mouse treated with cycloheximide followed by urethan (2 mg/g body weight) and killed 1.5 hr after the last injection. Digestion with Pronase on ultrathin section after previous H2O2 oxidation. Microspherules (arrow) are completely digested. Uranyl acetate and lead citrate, × 29,000.
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