Biosynthesis of DNA, RNA and proteins by mouse embryos cultured in the presence of a teratogenic dose of chlorambucil

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SUMMARY

The effect of chlorambucil on the rates of DNA, RNA, and protein synthesis in mouse embryos was investigated using a system of whole embryo culture. Embryos were isolated on the 11th day of gestation (33 ± 3 somites) and grown in culture media for periods of 4–8 h. Reichert's membrane and most of the placental tissue was removed leaving only the amnion and visceral yolk-sac surrounding the embryo. In the presence of teratogenic doses of chlorambucil (15 µg/ml) the rate of DNA synthesis was significantly decreased at 4 and 8 h. RNA and protein synthesis were not inhibited at either of these times. A trend toward decreasing rates of protein synthesis at some time beyond 8 h was noted, but not tested.

INTRODUCTION

Prior studies have established the teratogenicity (Didock, Jackson & Robinson, 1956; Murphy, Moro & Lacon, 1958; Murphy, 1959; Monie, 1961; Chaube & Murphy, 1968; Sadler & Kochhar, 1975) and cytotoxicity (Sadler & Kochhar, 1976) of the alkylating agent chlorambucil in rat and mouse embryos. In developing mouse limbs, the drug produced numerous deformities and caused large amounts of necrosis after treatment in vivo or in vitro (Sadler & Kochhar, 1975, 1976). The exact relationship between the pattern of cell death and observed malformations is not known, but limb-buds with large numbers of dead cells develop more severe limb defects.

After exposure to chlorambucil, affected cells are eliminated by autophagy, but the biochemical lesion responsible for the cell death is not understood. Short, Rao & Gibson (1972) have reported inhibition of in vivo DNA synthesis in mouse embryos following administration of a similar alkylating agent cyclophosphamide, and Ritter, Scott & Wilson (1971, 1973; Scott, Ritter & Wilson, 1971, 1973) have observed that inhibitors of DNA synthesis, such as

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cytosine arabinoside and hydroxyurea, produce severe necrosis in developing rat embryos. Brookes & Lawley (1960, 1961), as well as other investigators (Wheeler, 1962; Kohn, Spears & Doty, 1966; Lawley, 1966, 1967; Jolly & Ormerod, 1973), have reported that the DNA molecule is the site of action of these drugs. They have shown in cell cultures and cell-free systems that, as bifunctional alkylating agents, chlorambucil and cyclophosphamide can crosslink DNA by forming a covalent linkage between two opposing guanine molecules. However, it has not been determined if this mechanism is responsible for the drugs’ cytotoxicity in embryonic systems.

The studies described above suggested that if the drug acted by crosslinking DNA, then DNA synthesis should be inhibited prior to any effect on the synthesis of RNA or protein. Therefore, this study was designed to examine the effect of chlorambucil on DNA, RNA, and protein synthesis in embryonic mouse tissue and to relate any effect to the observed cell death and malformations produced by the drug.

**MATERIALS AND METHODS**

Pregnant random-bred mice of ICR/DUB strain (Flow Laboratories, Dublin, Virginia) were killed on the 11th day (plug day = 1st day) of gestation and both uterine horns were removed and placed in sterile Tyrode’s solution. Embryos were stripped from the uterus, taking care to leave Reichert’s membrane, the yolk-sac and the placenta intact. Each embryo was then placed in a watch glass containing just enough Tyrode’s solution to submerge the embryo. Then, under the dissecting microscope, Reichert’s membrane was carefully removed leaving the visceral yolk-sac intact. A major portion of the placenta was also trimmed away leaving some tissue attached to the allantoic vessels. After observing the general pattern of yolk-sac circulation, two tears, one approximately 0.5 cm and the other 0.2 cm in length, were made in the visceral yolk-sac at the cephalic and caudal ends respectively. Care was taken not to damage the embryo, amnion, or major blood vessels traversing the yolk-sac. Finally, to remove excess blood cells, each embryo was washed twice in Tyrode’s solution and then placed in a sterile 10 ml screw-capped culture vial containing 2 ml of medium. The vials were gassed with a mixture of 5% CO₂ and 95% oxygen, capped tightly, placed on a rotator at 37 °C, and rotated at 36 rev/min for periods of up to 8 h.

Culture medium consisted of 50% Waymouth’s solution (Flow Laboratories) and 50% fetal calf serum (Flow Laboratories) to which had been added 12.5 μg/ml streptomycin and 7.5 μg/ml penicillin G. After 1 h on the rotator to permit acclimatization to the culture system, heartbeats and circulation were monitored. At this time some cultures received 30 μg of chlorambucil in 10 μl absolute ethanol (final concentration was 15 μg/ml). Control cultures received only absolute ethanol at this time. Only cultures having strong heartbeats (172–240 beats/min) and grade 3+ (on a scale of 0–3+) circulation after the
acclimatization period were used in the study. Circulation was graded according to the degree of flow of red blood cells through the yolk-sac and embryo, with 0 being no flow and 3+ being rapid flow.

RNA synthesis was measured by adding 1 \( \mu \)Ci/ml of \([\text{3H}]\)uridine (sp. act. 8 Ci/mmole) in 10 \( \mu l \) BGJ (Bigger's solution) to both treated and control vials at the same time that chlorambucil was added. Cultures were maintained for 4 or 8 h, and the heartbeats and circulation were again monitored with the elimination of cultures not meeting the standards already described. At the end of the culture period the vials were removed from the incubator and placed in an ice bath for 5 min to decrease the rate of circulation. Embryos were then removed, washed twice in saline solution, and placed in another dish of saline under a dissecting microscope. The visceral yolk-sac and placenta were removed and the embryos were washed in fresh saline and then placed in centrifuge tubes and stored overnight at \(-50^\circ C\).

RNA was extracted by homogenizing each embryo in 1 ml cold (4 \(^\circ C\)) distilled \( H_2O \). An equal volume of cold 10% trichloroacetic acid (TCA) was added and the resultant precipitate was centrifuged at 4 \(^\circ C\) for 10 min at 12000 \( g \). The precipitated material was washed twice with cold 5% TCA, and RNA was extracted by the addition of 1 ml of cold (4 \(^\circ C\)) 10% perchloric acid (PCA). After storage overnight at 4 \(^\circ C\), the sample was centrifuged at 4 \(^\circ C\) for 10 min at 12000 \( g \). The PCA supernatant containing RNA was saved and the pellet was washed with an additional 1 ml of cold (4 \(^\circ C\)) 10% PCA, followed by centrifugation at 4 \(^\circ C\) for 10 min at 12000 \( g \). Both supernatants were combined and a 0.2 ml aliquot was counted for radioactivity on a liquid scintillation counter (Isocap 300, Nuclear Chicago) equipped with an external standard system. Total RNA content was measured by reading the remainder of the supernatant against a standard (yeast RNA type XI, Sigma, St Louis, Missouri) at 260 m\(\mu\) on a Gilford model no. 240 spectrophotometer (Ruddick & Runner, 1974).

DNA synthesis was measured in the same way as RNA with the exception that 2 \( \mu \)Ci/ml of [\text{3H}]thymidine (sp. act. 6 Ci/mmole) in 10 \( \mu l \) BGJ were added instead of uridine. Cultures were maintained and terminated as before. After termination, each embryo was placed overnight in 1 ml of cold (4 \(^\circ C\)) 10% PCA and then homogenized. The homogenate was centrifuged at 4 \(^\circ C\) for 15 min at 12000 \( g \) and then washed twice with 1 ml of cold (4 \(^\circ C\)) 10% PCA and centrifuged. DNA was extracted by hydrolyzing the precipitate in 1 ml of 10% PCA for 45 min at 90 \(^\circ C\). The sample was then centrifuged at 30 \(^\circ C\) for 15 min at 12000 \( g \), and the precipitate washed with 1 ml of hot 10% PCA (70 \(^\circ C\)), and centrifuged as before (Ellison & Lash, 1971). Both supernatants were combined and a 0.2 ml aliquot was counted for radioactivity by liquid scintillation, while 1 ml was saved for analysis of total DNA by the diphenylamine procedure (Burton, 1956), using calf thymus DNA as a standard (Sigma).

Protein synthesis was analyzed by adding 3 \( \mu \)Ci/ml of [\text{3H}]leucine (sp. act. 47 Ci/mmole) in 10 \( \mu l \) BGJ to each vial containing 50% fetal calf serum and
Table 1. Treatment and survival of embryos during whole embryo culture

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of embryos</th>
<th>Time in* (h)</th>
<th>Treatment</th>
<th>Survival after treatment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dead</td>
</tr>
<tr>
<td>1</td>
<td>64</td>
<td>4</td>
<td>Label + 10 μl EtOH</td>
<td>6.3</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>4</td>
<td>Label + 30 μg chlorambucil in 10 μl EtOH</td>
<td>3.7</td>
</tr>
<tr>
<td>3</td>
<td>46</td>
<td>8</td>
<td>Label + 10 μl EtOH</td>
<td>8.7</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>8</td>
<td>Label + 30 μg chlorambucil in 10 μl EtOH</td>
<td>15.6</td>
</tr>
</tbody>
</table>

* This time encompasses the period from treatment to termination of the cultures and does not include the acclimatization period.
† 3+ heartbeat, 3+ circulation. Only these were used in biochemical analyses.

50% Waymouth's medium which lacked leucine. Cultures were terminated as described before and the embryos were frozen for 24 h at −50 °C. Embryos were then homogenized in 1 ml of cold (4 °C) distilled H₂O. Protein was precipitated by the addition of 1 ml of 20% TCA for 24 h at 4 °C followed by centrifugation at 4 °C for 15 min at 12000 g. After decanting the supernatant, the precipitate was solubilized in 1 ml of 1 N-NaOH. A 0.2 ml aliquot was withdrawn for measurement of radioactivity with a liquid scintillation counter and another 0.2 ml was analyzed for protein content by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951) using bovine albumin (Sigma) as a protein standard.

All radioactive precursors were purchased from Schwarz/Mann, Orangeburg, New York.

RESULTS

A dose of 15 μg/ml chlorambucil was found to affect synthesis without killing many embryos. Doses above this level caused death of a large number of embryos and lower doses produced no effect on synthesis (Sadler, unpublished results). It was also found that 7% of the embryos died during the acclimatization period before any isotopes or drug was added. These deaths were attributed to surgical trauma during removal of the membranes which were sometimes difficult to peel off, especially in younger embryos (30 somites).

Table 1 indicates the effect of the drug and culture system on all embryos. More treated embryos died after 8 h in culture than after 4 h, whereas there was no difference in the number of deaths in controls at 4 or 8 h. On the other hand, embryos in good condition (3+ heartbeat, 172–240 beats/min, 3+ circulation) after treatment continued to be so at 4 or 8 h. Thus, the percentage of treated embryos in good condition did not differ from that at 8 h, nor did significant differences occur in controls at 4 or 8 h. Finally, there was a higher
Fig. 1. Comparison of uptake of tritiated precursors of DNA, RNA, and protein in control and treated (chlorambucil, 15 μg/ml) embryos grown in culture. Differences in the amount of incorporation of label into DNA in control vs treated embryos are significant (P < 0.01). No significant differences were found for RNA or protein.

percentage of control and treated embryos in poor condition at 4 h than at 8 h. In controls, this could perhaps be due to increased recovery from surgery; in the treated embryos, some of those in poor condition at 4 h may have died by 8 h.

Figure 1 shows the rate of uptake of radioactive precursors into DNA, RNA, and protein. These data were analyzed by an unweighted means analysis of variance for unequal numbers (Meyers, 1941). Treated embryos showed lower rates of [3H]thymidine uptake into DNA than controls, and the level of uptake was greater at 8 h than at 4 h. The interaction of time and treatment was also significant at < 0.01, such that the increase in rate of [3H]thymidine uptake from 4 to 8 h was greater in control than in treated embryos. Analysis of RNA and protein synthesis showed no significant effect of treatment, but in both treated and control cultures the rate of synthesis increased from 4 to 8 h (P < 0.01). This indicates that the system is capable of detecting time differences, so the lack of treatment effect is not merely due to the variance inherent in the system. There was no significant time × treatment interaction for RNA or protein, indicating that no differential effect on synthesis was found between treated embryos versus controls at 4 or 8 h.
DISCUSSION

Several techniques for the culture of postimplantation rat and mouse embryos have been devised (Cockroft, 1973; New, 1973; New, Coppola & Terry, 1973). The present study employed a modification of techniques developed by New and Cockroft. Basically, the culture system was of New’s design, but instead of placing culture vials on rollers, they were placed on a model 150 V rotator (Scientific Industries, Springfield, Mass.) and rotated at 36 rev/min (vs 60 rev/min in New’s system). A further modification of New’s procedure was made by tearing the visceral yolk-sacs in the manner described by Cockroft (the amnion was left intact which differed from Cockroft’s procedure). This was done to expose the embryos directly to the teratogen, since previous studies had revealed that the drug had little effect when the yolk-sacs were left intact (Sadler, unpublished results). Cultured in this fashion, the teratogenic effect of the drug on the embryo could be examined directly.

Embryos cultured in this system can be maintained for 40 h with continued growth and development (Kochhar, 1975). For example, embryos placed in culture on the 11th day of gestation (30 somites) develop to the 40–42 somite stage by 40 h. This amount of development encompasses critical stages in the period of organogenesis, particularly early limb development, which was of primary interest. A period of 8 h in culture was selected for testing in this study because this amount of time was short enough for efficient embryonic growth throughout the culture period. It was also long enough to study the initial biochemical effects of such a rapidly acting drug as chlorambucil.

The results indicate that control embryos that survived the trauma of surgery and the acclimatization period were maintained quite well by the system (Table 1), as measured by the steady increase in DNA, RNA, and protein synthesis (Fig. 1). That the embryos were surviving well was also indicated by their rates of heartbeats and circulation, which were maintained or increased from the initial rates after acclimatization.

The results also show that the first effects of treatment with chlorambucil are reflected in decreased rates of DNA synthesis as early as 4 h following treatment. Neither protein nor RNA synthesis was significantly inhibited during the 4 or 8 h culture periods, although there was some indication that protein synthesis may be inhibited after 8 h. These findings are consistent with those of Short et al. (1973), who studied the in vivo rates of DNA, RNA, and protein synthesis in mice after treatment with another nitrogen mustard, cyclophosphamide, on day 11 of gestation. They found that DNA synthesis was reduced 12 h after administration of the drug and total DNA content was reduced 12, 24, 48, and 72 h after treatment. Protein synthesis was not significantly reduced until 72 h after treatment and RNA synthesis was not altered.

All of these findings are consistent with the results of Brookes & Lawley (1961) and other workers (Wheeler, 1962; Kohn et al. 1966; Lawley, 1966;
Jolley & Ormerod, 1973) who have proposed that the nucleus and DNA are the site of action of nitrogen mustards. They have found that these agents, including chlorambucil, have the capacity to alkylate (crosslink) DNA in a covalent linkage. The formation of crosslinks in the DNA molecule interferes with transcription which eventually may lead to cell death. Although the present study does not examine embryonic DNA for crosslinks, the results indicate that synthesis of this nucleic acid is the first to be affected by the drug, whether through crosslinking or some other mechanism, and suggest that defects in DNA may be responsible for the cytotoxic effects of chlorambucil. Furthermore, the first histological evidence of the drug's effect is at 4 h (Sadler & Kochhar, 1976), which coincides with significant inhibition of DNA synthesis at 4 h. However, the alkylating agents are known to affect many cell functions and without further biochemical investigation it is not possible to identify a direct effect on DNA.

The results are also important for two other reasons: First, since they agree with Short's results using in vivo techniques they suggest that this group of agents (nitrogen mustards) must act on the embryo directly rather than through intermediation of the maternal system. Although cyclophosphamide must be activated by the maternal liver, it is the alkylating portion of the molecule which becomes active and it is this same chemical group which is active in chlorambucil. Therefore, it is logical to suggest that the maternal system plays no role in altering this portion of the molecule since the same effects are produced in vivo by cyclophosphamide or in vitro by chlorambucil. Further evidence to support this hypothesis can be derived from the fact that, morphologically, cells exposed to the drug in vivo, i.e. through the maternal system, show the same adverse effects that occur in cells from tissues exposed in vitro (Sadler & Kochhar, 1976). Thus, the type of chlorambucil-induced cell death is identical in vivo or in vitro indicating that the drug acts the same way in both systems and is not significantly altered by the mother. Secondly, these two agents cannot be acting by affecting yolk-sac or placental function since the in vitro technique of tearing the yolk-sac removes this barrier normally present between the embryo and drug, and the same results, i.e. inhibition of DNA synthesis, are incurred whether the yolk-sac and placenta are intact (in vivo) or not (in vitro).

Finally, the study shows that this type of culture system is sensitive enough to detect biochemical differences produced by exposing embryos directly to teratogens. It therefore provides a means of bypassing the maternal system, with all its variables, and studying the direct effects of teratogens on embryonic development during the period of organogenesis.

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