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THE EFFECTS OF LASIOCARPINE, RETRORSINE AND RETRONECINE PYRROLE ON HUMAN EMBRYO LUNG AND LIVER CELLS IN CULTURE

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Summary.—Retronecine pyrrole induces toxic changes both in human liver and lung cells. Lasiocarpine and retrorsine are toxic to liver cells but not to lung cells, which are unable to metabolize the pyrrolizidine alkaloids to pyrroles. The application of lasiocarpine to human liver cells in culture is followed by inhibition of DNA, RNA and protein synthesis; vacuolation of the cells, the prevention of mitosis and the formation of giant cells (" megalocytes ").

THE pyrrolizidine alkaloids are alkylating agents which are found in a variety of common plants, notably species of Senecio, Heliotropium and Crotularia. These compounds have been implicated in the aetiology of both human and animal liver disease. Damage to the lungs may be a complicating factor (Selzer, Parker and Sapeika, 1951; Barnes, Magee and Schoental, 1964). These alkaloids are not very active chemically and it has been suggested that the microsomal enzymes of the liver activate the alkaloids, forming short-lived toxins. These metabolites induce severe liver damage and may reach the lungs *via* the bloodstream, inducing vascular and pulmonary lesions. Mattocks (1968) demonstrated the presence of pyrroles, which are highly reactive alkylating agents, in the liver and urine of rats previously exposed to hepatotoxic pyrrolizidine alkaloids. The production of pyrroles from lasiocarpine, heliotrine and fulvine by human embryo liver tissue has been demonstrated *in vitro* (Armstrong and Zuckerman, 1970). Human embryo lung tissue did not produce detectable amounts of pyrroles.

Cultures of human embryo liver and lung cells were exposed to 2 pyrrolizidine alkaloids, lasiocarpine and retrorsine, and to retronecine pyrrole, a possible metabolite of retrorsine. The tissue cultures were examined by light microscopy and by autoradiography.

MATERIALS AND METHODS

Primary human embryo cell cultures.—Foetal livers and lungs were obtained by the Tissue Bank of the Royal Marsden Hospital from normal human embryos after abdominal hysterotomy of women free from apparent infection. The age of the embryos varied between 10–18 weeks. The method of tissue culture of the liver has been described fully elsewhere (Zuckerman, Tsiquaye and Fulton, 1967) and a similar technique was used for the culture of lung.

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Exposure of the cultures to alkaloids.—Retrorsine hydrochloride is soluble in growth medium. Dilutions were prepared so that 0·5 ml of growth medium contained the required quantity of the alkaloid.

Lasiocarpine was dissolved in a few drops of dimethyl formamide (DMF), added to the growth medium, and serially diluted.

Retronecine pyrrole is highly reactive and it is inactivated 1–2 min after addition to the growth medium. Therefore, dilutions were made in DMF so that 1 drop of DMF contained the required quantity of the toxin and this was added to 0·1 ml of growth medium in the well. After 5 min the growth medium and DMF were replaced by fresh medium.

Radioactive Labelling and Autoradiography.—The radioactive materials used were 6[^{3}H]-thymidine (specific activity 5·0 Ci/mM), [^{3}H]-uridine (9) (specific activity 2·64 Ci/mM) and [^{3}H]-L-leucine (9) (specific activity 200 mCi/mM). These were supplied by the Radiochemical Centre, Amersham, Bucks. The tritated precursors were diluted in the growth medium to 0·5 μCi/ml. Leucine deficient medium was used for studies of protein synthesis. Cultures were exposed to the labelled precursors for 1 hour, then the cultures were fixed and prepared for autoradiography as described by Zuckerman *et al.* (1968).

The proportion of the cells which were synthesizing DNA during the hour of exposure was determined by counting the labelled and unlabelled nuclei under the light microscope. Cells which had been exposed to tritiated leucine or uridine were photographed, the transparencies were projected on to squared paper and the number of grains per cell counted. The treated and control cultures were compared and the results expressed as the percentage reduction of incorporation of the precursor.

Primary liver cell cultures are mixed cultures containing type I and II cells, which resemble hepatocytes, and type III cells which are elongated and resemble fibroblasts. Type I and II cells were counted separately from type III cells. Only hepatocytes were photographed for the estimation of uridine and leucine incorporation. Primary lung cell cultures contained 2 types of cells but these rapidly became indistinguishable and no attempt was therefore made to count the cell types separately.

RESULTS

The cultures were examined by fluorescence microscopy after staining with acridine orange. Liver cells exposed to 25 μg of any of the 3 alkaloids for 3 days showed similar morphological changes, which included loss of the orange fluorescence due to the presence of RNA, some vacuolation of the cytoplasm and occasionally of the nucleus, loss of definition of the cytoplasmic membranes and enlargement of some of the cells. Mitosis was inhibited. A small proportion of cells died but cultures treated with as much as 150 μg of lasiocarpine survived.

Lung cells appeared normal after incubation with 100 μg lasiocarpine or retrorsine for 3 days, or 25 μg for 7 days. However, 25 μg retronecine pyrrole induced morphological changes in lung cells which were similar but less pronounced than those in liver cells.

Autoradiography. The Effect of Increasing Amounts of Alkaloid

Autoradiography of lung cells using tritiated thymidine showed that retrorsine and lasiocarpine had no effect on DNA synthesis, confirming that lung cells are not susceptible to these pyrrolizidine alkaloids. However, liver cells showed inhibition of both DNA and RNA synthesis after treatment with retrorsine and lasiocarpine. The degree of inhibition increased rapidly as the quantity of alkaloid increased to 25 μg, but 50 and 100 μg did not increase markedly the inhibition. The graphs representing the depression of RNA and DNA synthesis after incubation of cultures with lasiocarpine and retrorsine are similar in shape (Fig. 1–3). DNA synthesis in type III cells was significantly less inhibited than in type I and

II after exposure to 25 μg lasiocarpine ($P < 0{\cdot}01$) and 50 μg lasiocarpine levels ($P < 0{\cdot}05$).

Retronecine pyrrole inhibited DNA synthesis in both liver cells and lung cells. There was no significant difference between the inhibition of synthesis in the 2 types of cultures or between type I and II and type III cells. If the values for

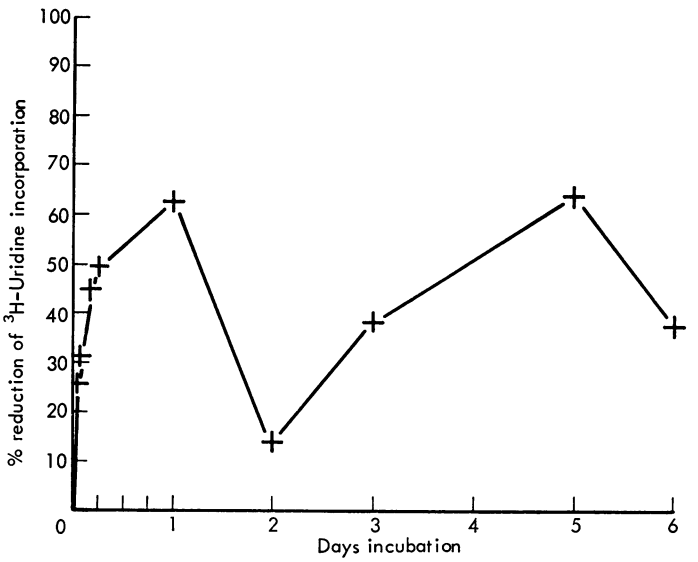

FIG. 1.—Effect of 25 μg lasiocarpine on RNA synthesis of hepatocytes.

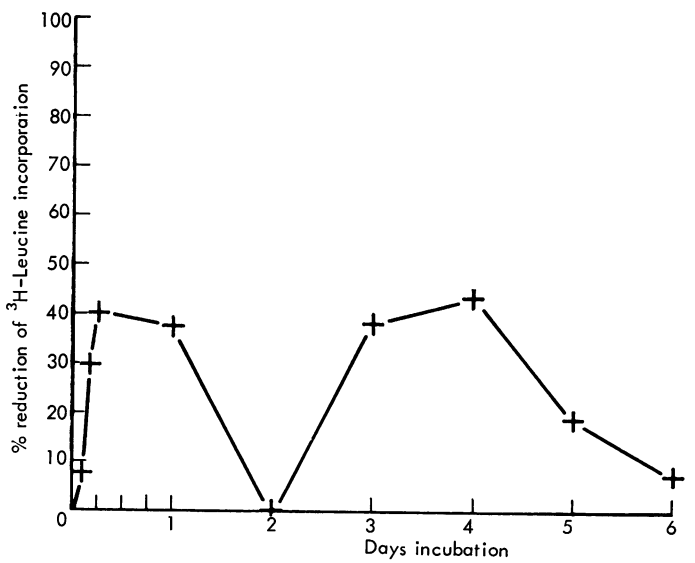

FIG. 2.—Effect of 25 μg lasiocarpine on protein synthesis of hepatocytes.

the inhibition of DNA synthesis are adjusted so that they are comparable to retrorsine with respect to the molecular equivalents, then the graphs for retrorsine and its metabolite, retronecine pyrrole, are found to be very similar in shape.

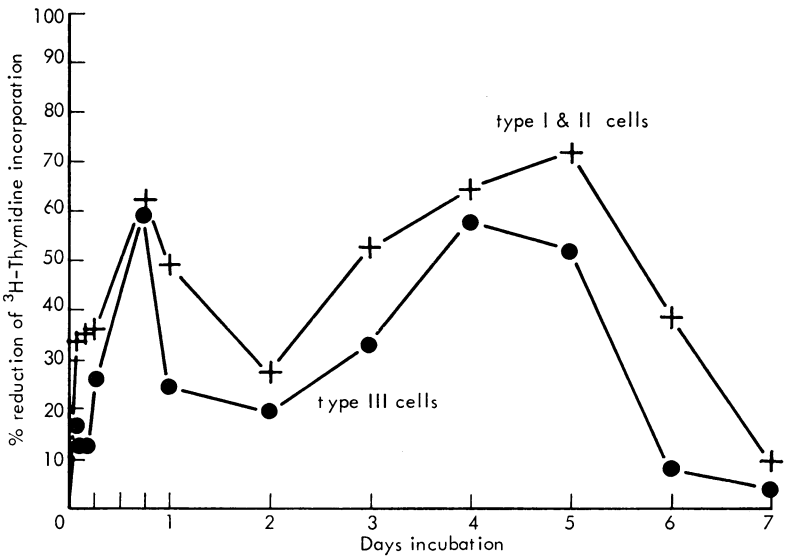

FIG. 3.—Effect of 25 μg lasiocarpine on DNA synthesis of foetal liver cells.

The Effect of Exposure to the Alkaloids for Various Periods of Time

Each culture was incubated with 25 μg of alkaloid and exposed to a tritiated precursor after a period ranging from 0 hours to 4 weeks. The growth medium was changed as necessary during the longer experiments.

The effect of lasiocarpine on DNA, RNA and protein synthesis was found to be similar. There were 2 major peaks of inhibition of DNA synthesis, one maximal at 18–24 hours and another at 4–5 days. There was also a small initial peak of inhibition, the timing of which varied between 2 and 4 hours in different experiments.

The initial small peak of inhibition of protein synthesis was significantly delayed ($P < 0{\cdot}02$) with respect to the inhibition of nucleic acid synthesis. The depression of uridine incorporation slightly preceded depression of thymidine incorporation. The maximal inhibition of protein synthesis in the third period occurred at 4 days, whereas the maximum inhibition of RNA and DNA synthesis occurred at 5 days. DNA synthesis was examined 1, 2, 3 and 4 weeks after exposure to 25, 50, 100 and 150 μg lasiocarpine. Synthesis was normal after incubation for 1 week with 25 or 50 μg of lasiocarpine, and by the third and fourth weeks all the cultures were normal in this respect. However there was 25 to 50 per cent inhibition of mitosis in these cultures after 4 weeks and many cells were abnormally enlarged.

The inhibition of RNA and DNA synthesis by retrorsine occurred in 2 phases, one maximal at 4–6 hours and one at 3–5 days. In individual experiments, it was

found that RNA synthesis was affected first. After incubation with 25 μg retrorsine and tritiated uridine for 1 hour there was 15 per cent inhibition of incorporation of the precursor. DNA synthesis was not affected. After 2 hours, RNA synthesis was inhibited by 48 per cent and DNA synthesis by 36 per cent. There was no peak corresponding to the second peak induced by lasiocarpine; in fact, RNA synthesis had returned to normal after 24 hr. Over a 7-day period there was no significant difference between the patterns of inhibition of DNA and RNA synthesis

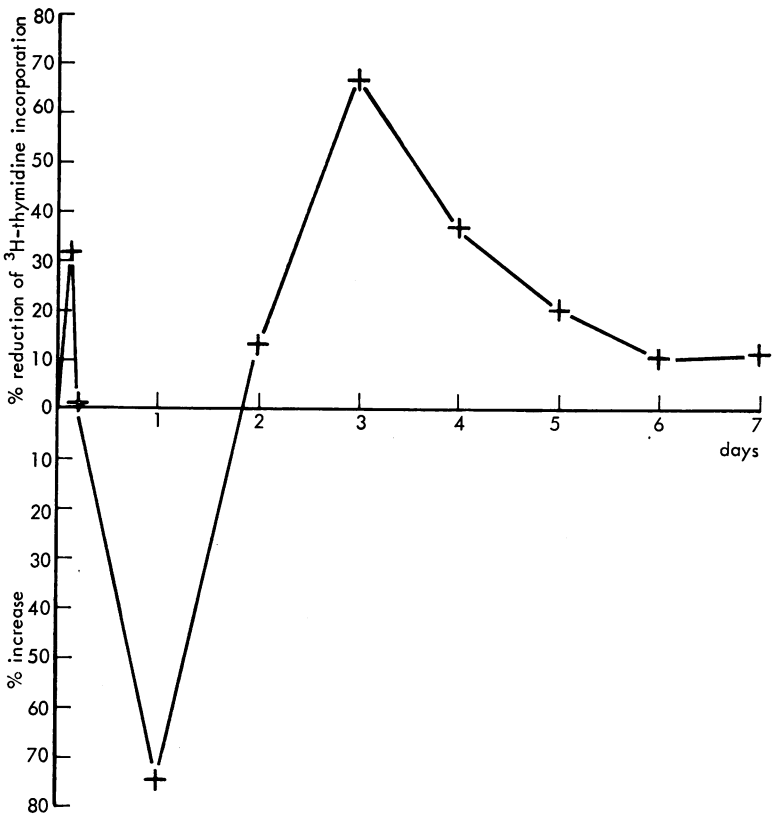

FIG. 4.—The effect of retronecine pyrrole on DNA synthesis in lung cells in culture (25 μg).

and no difference between the effect on DNA synthesis by type I and II and type III cells over the same period, although type III cells were less sensitive than hepatocytes to lasiocarpine ($P < 0{\cdot}05$).

The effect of retronecine pyrrole on the uptake of tritiated thymidine was unexpected. Treated lung cells showed 2 phases of inhibition of DNA synthesis. These were maximal 3 hours after the application of 25 μg pyrrole and on the 3rd day (Fig. 4). At 24 hours there was a large increase in the uptake of tritiated thymidine. However, there was no corresponding increase in RNA synthesis and no nuclear enlargement.

The pattern of incorporation of tritiated thymidine by liver cells was different. There was a small increase in incorporation between 22 and 48 hours, followed by marked depression of DNA from the third to at least the seventh day. There was apparently no increase in DNA synthesis in hepatocytes at 4 hours, although there was 30 per cent inhibition in type III cells at this time.

DISCUSSION

Lasiocarpine and retrorsine have previously been shown to be metabolized by human liver tissue but not by lung tissue (Armstrong and Zuckerman, 1970). Experiments with tissue cultures show that human embryo lung cells are not affected by these alkaloids, but under similar conditions mitosis and DNA, RNA and protein synthesis are depressed in foetal hepatocytes and these cells may undergo necrosis. Retronecine pyrrole is a probable metabolite of alkaloids such as retrorsine, which contain retronecine. This pyrrole is toxic to both liver and lung cells. It seems likely that the pyrrolizidine alkaloids are not hepatotoxic but that the liver can activate these compounds by conversion to pyrroles, which are toxic. Pyrroles are rapidly inactivated by combination with water molecules and the effects would thus be limited. Retrorsine causes more extensive lung damage *in vivo* than lasiocarpine, probably because the pyrrole products of retrorsine remain active for a longer time. This is suggested by the tissue culture experiments as the type III cells, which are fibroblastic in appearance, are less affected by exposure to lasiocarpine than the hepatocytes which are presumably producing and releasing the metabolite. However, the 2 cell types show similar inhibition of nucleic acid synthesis when retrorsine is applied.

Black and Jago (1970) demonstrated that dehydroheliotridine will bind calf thymus DNA *in vitro* to form a soluble complex. Dehydroheliotridine is the major pyrrole produced by rat liver *in vitro* from lasiocarpine and is almost identical in structure to retronecine pyrrole. It is probable that the metabolite binds to DNA and inhibits DNA and RNA synthesis, and that the DNA repair mechanism excises alkylated bases. Inhibition of protein synthesis follows later, but occurs more quickly than would be expected if the inhibition were merely a result of the lack of messenger RNA. Harris, Redy, Chiga and Svoboda (1969) found disaggregation of the polyribosomes in rat liver after injection of lasiocarpine and suggested that there might be an inhibition of the re-attachment of ribosomes to mRNA after translation.

The inhibition of mitosis is apparently not related directly to the inhibition of DNA synthesis. DNA synthesis recovers after a week, but mitotic inhibition was evident in cultures of a continuous liver cell line for at least 4 weeks. Giant cells were frequently seen and these synthesized DNA but presumably were unable to divide. The giant cells appear to be analagous to the megalocytes seen *in vivo* (Bull, 1955).

The initial phase of inhibition may be due to alkylation of DNA and corresponds to changes in the appearance of the chromatin and of the nucleoli. The major peak of inhibition of nucleic acid synthesis seems to be an indirect effect since it is preceded by inhibition of protein synthesis. This may be due to the delayed effects of the alkylation of essential enzymes and structural components of the cell which would lead to a reduced metabolic rate and a decreased level of synthesis of cell components.

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