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# PRELIMINARY FINDINGS OF CHROMOSOMAL STUDIES ON RATS AND HUMANS WITH VENO-OCCLUSIVE DISEASE

PATRICIA A. MARTIN*, MARIGOLD J. THORBURN, SYBIL HUTCHINSON, G. BRAS AND C. G. MILLER

*From the Department of Pathology, University of the West Indies, Mona, Kingston, Jamaica and the Department of Paediatrics, University of the West Indies*

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**Summary.—Peripheral blood chromosome studies were performed on 9 children in the acute and recovery phase of veno-occlusive disease (VOD) of the liver, which is thought to be due to the toxic effect of fulvine, an alkaloid in *Crotalaria fulva* a plant consumed as a herbal remedy in Jamaica. Similar studies were performed on rats in which VOD was induced by the experimental administration of fulvine. Significant percentages of mitotic cells showing chromosomal damage in the form of gaps, breaks, dicentrics and exchanges were found in 6 children tested within 3½ months from the time of ingestion of *Crotalaria fulva* and in rats up to 15 days after administration. This nuclear damage may correspond to the mutagenic effect observed in other pyrrolizidine alkaloids and the nuclear abnormalities observed in liver cells damaged acutely by fulvine and lasiocarpine.**

VENO-OCCLUSIVE disease of the liver (VOD) is an important clinical disease in Jamaica, leading to cirrhosis in some cases (Bras, Brooks and Watler, 1961). *Crotalaria fulva*, one of the plants responsible for causing VOD, has as its active principle a hepatotoxic pyrrolizidine alkaloid, fulvine. The pyrrolizidine series of alkaloids is of considerable theoretical and practical interest because of the range of their biological activities. They have been reported to cause mutations in *Drosophila melanogaster* (Clark, 1959; Cook and Holt, 1966) and to produce chromosomal breakage in plants (Avanzi, 1961) and in rat intestinal epithelium (stated by Culvenor *et al.*, 1962). Induction of liver tumours and teratogenic effects in rats have been reported by Schoental and Head (1955) and Green and Christie (1961) respectively.

Chromosomal aberrations may accompany carcinogenic, mutagenic and teratogenic properties in many compounds. In view of these observations, it was thought that cytogenetic studies might provide interesting information in patients and rats with VOD. This paper reports a preliminary study of rats given doses of fulvine sufficient to produce VOD and studies on 9 children with recent or recovered VOD.

## MATERIALS AND METHODS

*Fulvine.*—Solutions of fulvine were freshly prepared by adding the purified crystalline alkaloid to sterile distilled water and sufficient hydrochloric acid to adjust the pH to 7·4.

*Rats.*—Mature well nourished male and female albino rats weighing 200–210 g were used

* Present address: Department of Medical Genetis, McGill University, Montreal, P.Q., Canada.

Requests for reprints to Dr M. J. Thorburn, Department of Pathology. University of the West Indies.

for all experiments. They were kept in individual cages and fed standard Purina Chow and water *ad libitum*. Blood was removed from the tail vein or by cardiac puncture at the time of sacrifice.

*Patients.*—In the *in vitro* studies, blood from random volunteers was used. In the *in vivo* studies there were 9 children, seen in the paediatric wards or clinics of the University Hospital of the West Indies with biopsy proven VOD. Seven patients were in the acute stage of the disease and 2 had recovered. Clinical details are shown in Table I. There was no history of exposure to known mutagens. Parents and siblings without evidence of VOD, normal volunteers and 2 children with whooping cough served as controls.

*In vitro studies*

These experiments were performed on rat fibroblasts and rat and human peripheral blood lymphocytes. Rat fibroblasts were cultured by the method of Soukup (personal communication). At second subculture, fulvine was added to the medium at a final concentration of 50, 100, 150 and 250 $\mu$g/ml for 24 hours. Two cultures were treated at each dosage level. Two sets of peripheral blood cultures from normal rats and normal humans were each treated with doses of 60, 100, 150 and 300 $\mu$g/ml for 48 hours. Control specimens received the same volume of dilutant.

*In vivo studies*

*Rat.*—Fulvine was injected intraperitoneally in a dosage of 0·08 mg/g body weight; 2 rats received subcutaneous administration. Controls were given the same volume of dilutant. They were killed at daily intervals after treatment and also had blood taken from the tail for chromosome studies. The liver was removed at sacrifice for histological examination.

*Human.*—Peripheral blood was drawn at varying intervals after development of the disease, as shown in Table I.

TABLE I.—*Clinical Data. Relationship of Ingestion of "Bush Tea" to Onset of Disease and Cytogenetic Study*

| Patient | Sex and Age | Date of Ingestion | Period of Ingestion | Onset of VOD | Ingestion-sampling interval |
|---|---|---|---|---|---|
| P.C. | F 3 | 23.5.69 | 2–3 weeks | 15.6.69 | 6 weeks |
| Do C | F 10/12 | 18.5.69 | 2–3 weeks | 8.6.69 | 6 weeks |
| Di C | F 2 | 12 5.69 | 2–3 weeks | 14.6.69 | 6 weeks |
| G.S. | M 9/12 | 25 3 69 | 3 days | 15.4.69 | 3½ months |
| M.S. | F 2 | 25.3.69 | 3 days | 19.4.69 | 3½ months |
| D.M. | M 2½ | March, 1969 | 2 days | May, 1969 | 3 months |
| K.B. | F 2½ | May, 1968 | ? | June, 1968 | 16 months |
| L.M. | F 12 | May, 1968 | ? | June, 1968 | 16 months |
| I.W. | F | Denied | ? | Oct., 1969 | 1 month |

TABLE II.—*In Vivo Production of Chromosome Anomalies in Rats with VOD*

| Day tested | No. of cells examined | No. of gaps | No. of breaks | Other configurations | % Abnormal cells |
|---|---|---|---|---|---|
| 3 | 52 | 2 | 2 | 4 | 9·6 |
| 4 | No mitoses | | | | |
| 5 | 50 | 2 | 2 | 2 | 10 |
| 6 | 50 | 1 | 3 | 2 | 11·3 |
| 7 | 54 | 1 | 2 | 5 | 24 |
| 8 | 19 | 1 | 9 | 1 | 20 |
| 15 | 54 | 8 | 7 | 7 | 24 |
| 17 | 35 | 1 | 0 | 2 | 6·6 |
| 20 | No mitoses | | | | |
| 24 | 50 | 1 | 1 | 0 | 4 |
| 30 | 50 | 0 | 2 | 0 | 4 |
| Controls | | | | | |
| 8 | 104 | 0 | 3 | 0 | 3 |
| 15 | 50 | 1 | 0 | 0 | 2 |
| 35 | 29 | 1 | 0 | 0 | 3 |

*Cytogenetic technique*

Peripheral blood was cultured by a modification of the microtechnique of Arakaki and Sparkes (1963). Chromosome spreads were selected under low magnification (×200), and analysed under oil immersion (×800). Standard procedure for scoring structural abnormalities (Court-Brown, 1967) was employed. Cells from the patients were karyotyped using the Denver classification.

## RESULTS

The *in vitro* studies were negative in all experiments though there was some chromosome fuzziness and stickiness.

The findings from the *in vivo* studies in rats are shown in Table II. The mitotic rate in the experimental animals was lower than in the controls. Multiple breaks and gaps of the chromatid and isochromatid type were found in many cells and there were frequent anomalous configurations (Fig. 1 and 2). The relationship of the percentage of cells with chromosome abnormalities to the time of sampling is shown in Fig. 3.

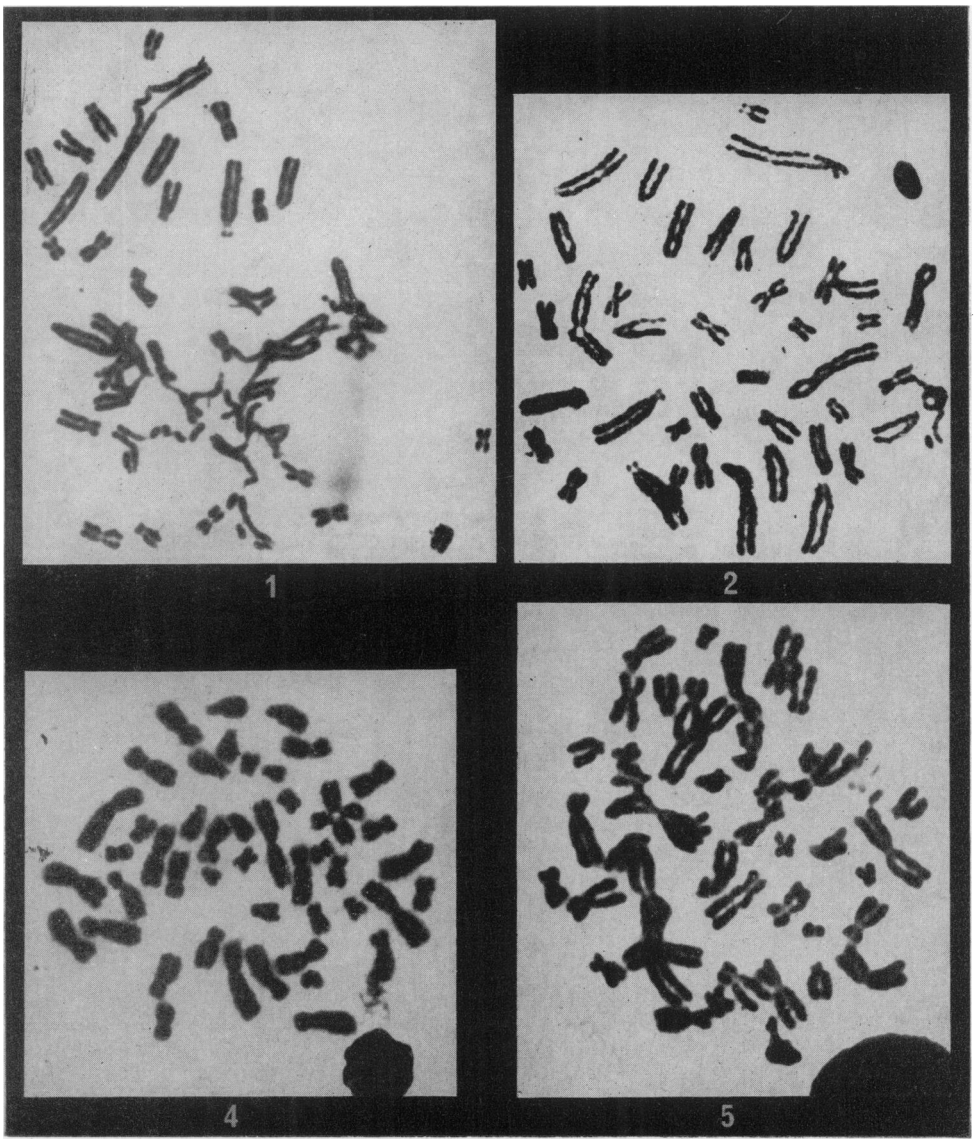
In the studies on humans, there was no significant difference in the mitotic rate between patients and controls. Abnormalities included gaps and breaks, dicentric forms and quadriradial configurations (Fig. 4 and 5). For the number of abnormal cells in cases P.C., Di. C. and D.C., the level of significance was impressive ($P < 0{\cdot}01$). There was a high frequency of breaks in chromosomes with secondary constrictions, namely numbers 1, 9, 13–15, and many of these chromosomes which were not broken had exaggerated or attenuated secondary constrictions. The details of chromosome counts and percentage of cells with breaks are shown in Table III and the types of anomalies found in Table IV. It will be noted that one patient, I.W., had no anomalies. This child died during the acute illness and the parents denied any ingestion of " bush tea " (medicinal herbs which may include *Crotalaria fulva*).

TABLE III.—*Percentage of Cells with Abnormalities in Patients and Controls*

| VOD Patients | | | Family controls | | | Pertussis patients | | |
|---|---|---|---|---|---|---|---|---|
| Patient | No. of cells analysed | % Abnormalities | Patient | No. of cells analysed | % Abnormalities | Patient | No. of cells analysed | % Abnormalities |
| P.C. | 52 | 60 | N.J. | 42 | 7·7 | D.P. | 25 | 0 |
| Do. C. | 50 | 40 | O.C. | 26 | 0 | W.S. | 52 | 0 |
| Di. C. | 65 | 28 | | | | | | |
| G.S. | 23 | 20 | | | | | | |
| M.S. | 6 | 17 | controls failed | | | | | |
| K.B. | 50 | 6 | D.M. | 26 | 0 | | | |
| L.M. | 21 | 5 | V.W. | 26 | 0 | | | |
| D.M. | 39 | 13 | control failed | | | | | |
| L.W. | 26 | 0 | M.C. | 26 | 0 | | | |

## EXPLANATION OF PLATES

FIG. 1 and 2.—Rat mitotic lymphocyte showing chromosomal breakage and exchanges.
FIG. 4.—Human cell showing a quadriradial configuration.
FIG. 5.—Human cell showing a dicentric chromosome.


1
2
4
5

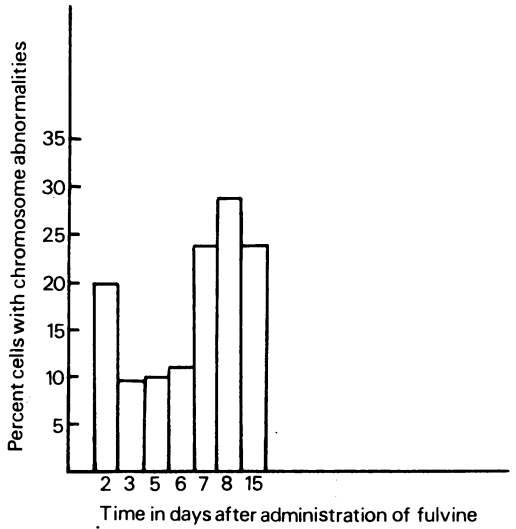



FIG. 3.—The percentage of cells showing aberrations in rats treated with fulvine.

TABLE IV.—*Analysis of Types of Abnormalities in VOD Patients*

| Patient | No. of cells | ASC | ICG | Breaks | Dicentrics | Anomalous configurations |
|---|---|---|---|---|---|---|
| P.C. | 52 | 8 | — | 13 | — | 5 |
| Di. C. | 65 | | 7 | 14 | — | — |
| Do. C. | 50 | 1 | 2 | 21 | 1 | 1 |
| G.S. | 23 | 3 | — | 1 | 1 | — |
| K.B. | 50 | 1 | 1 | 2 | — | — |
| L.M. | 21 | — | — | 1 | — | — |
| D.M. | 39 | 3 | 0 | 1 | 1 | — |

ASC Attenuated Secondary Constrictions
ICG Isochromatid gap.

The patients with the highest percentage of abnormalities were the 3 sibs of the C family. This family was tested the earliest in the course of the disease. This might suggest an inverse relationship of percentage of cells with abnormalities to the duration of time elapsing between ingestion and testing, though familial susceptibility cannot be excluded. Fig. 6 and 7 show this time relationship in all the patients and in the C family respectively.

## DISCUSSION

The results of this preliminary study are of interest in relation to the activities of the pyrrolizidine alkaloids and for the addition of, as far as we are aware, a new substance to the list of chromosome damaging agents.

The depressed mitotic rate in some of the early rat cultures is interesting. Inhibition of mitotic activity has been observed in the nuclei of hepatic parenchymal cells in liver damaged with lasiocarpine, another pyrrolizidine alkaloid (Peterson, 1965). Schoental and Magee (1959) suggested that these alkaloids may act as an interphase mitotic poison.

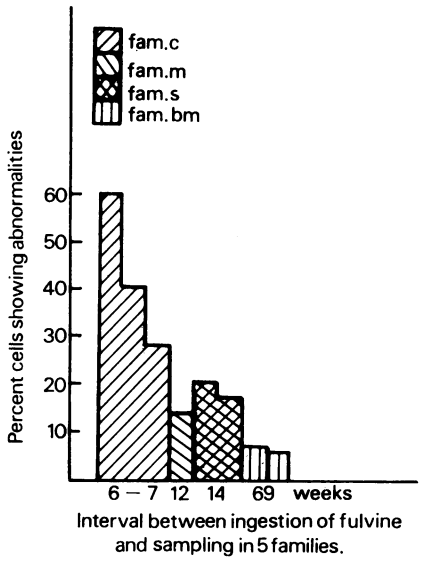



FIG. 6.—The percentage of cells showing abnormalities in 4 families, in relation to the time of sampling.

The non-random distribution of breaks observed is characteristic of chemically induced chromosome breakage. The dicentrics and quadriradial configurations, not found in controls, are similar to those demonstrated by Cohen, Hirschhorn and Frosch (1967) in cultured leucocytes from LSD " users ". The quadriradial

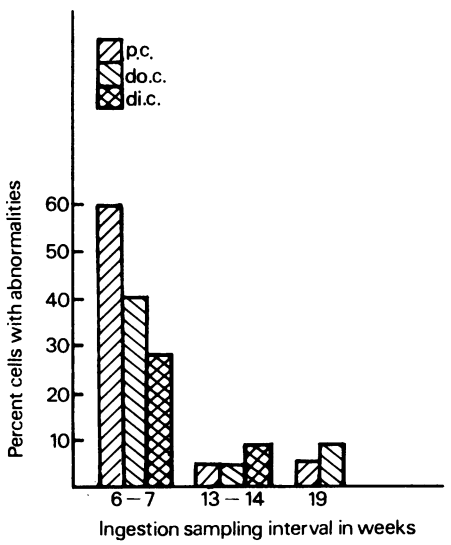



FIG. 7.—Family C showing the fall in percentage of chromosomal abnormalities as the time interval from ingestion of bush tea increased.

figures represent chromosomal exchanges, some resembling the pachytene cross configurations seen after a reciprocal translocation in meiosis. Most of the exchange figures apparently occurred between homologues or apparent homologues. The genetic consequences of these exchanges might be analogous to somatic crossing-over (Shaw and Cohen, 1965).

Nuclear abnormalities in liver cells have been reported in acute liver damage after the administration of fulvine and lasiocarpine (Gardiner, Royce and Bokor, 1965; Svoboda and Soga, 1966), though the latter found no changes in endothelial cells. Our study demonstrates that lymphocytes show mitotic irregularities after exposure to fulvine. In studies of the effects of acute (Peterson, 1965) and chronic (Schoental and Magee, 1959; Bull and Dick, 1959) pyrrolizidine alkaloid poisoning on liver cell division, it was shown that once the abnormalities are initiated they may not require the continued presence of the alkaloid for further development. In this study, chromosome abnormalities were observed up to 15 days after injection of fulvine in rats, whereas they were observed for as long as 14 weeks in some of the patients after ingestion of *Crotalaria fulva*. Circulating lymphocytes have a long intermitotic life span and chromosome damage of long duration in lymphocytes has been observed in children exposed to LSD *in utero* (Cohen *et al.*, 1968).

Gardiner *et al.* (1965) concluded that the pyrrolizidine alkaloids exerted their primary proliferative effect on cell types belonging to the reticulo-endothelial system. The chromosome damage induced in leucocytes in this study may also occur in these cells and result in the lesions observed by these investigators.

It is not yet apparent whether the lymphocyte chromosomal abnormalities are associated with the development of VOD or are a separate entity. The negative *in vitro* findings suggest that either VOD is necessary (in some unexplained way) for the appearance of abnormalities or (perhaps more likely) the chromosome damaging agent is a metabolite of fulvine. Jago (1969) suggested that both age and sex may influence the response to lasiocarpine, and it is of interest that of our 9 patients, 7 were female and 7 were less than 3 years old. The implications of our findings are of some importance. It is now well documented that there is an association between chromosome damage and a predisposition to neoplasia. As mentioned before, administration of other pyrrolizidine alkaloids has been shown to lead to liver tumours (Schoental and Head, 1955). There is also the possibility of a genetic effect if the gametes are exposed to the action of the alkaloid. Breakage and subsequent faulty healing of chromosomes may result in structural anomalies including balanced reciprocal translocations and other re-arrangements leading to teratogenesis or abortion. It is evident that further cytogenetic studies of VOD patients offer scope for investigation and long-term studies on recovered VOD patients are now in progress.

Further work on rats is also necessary to determine how long the chromosomal abnormalities persist, the minimal dosage of fulvine required to produce chromosomal damage, whether the abnormalities occur only in the presence of VOD, and whether chronic subclinical dosage results in lesions.

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