THE EFFECTS OF AFLATOXINS ON HUMAN EMBRYO LIVER CELLS IN CULTURE

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THE aflatoxins form a group of closely related substances produced by certain strains of *Aspergillus flavus-oryzae*. These substances have been shown to exert marked toxic and carcinogenic activities on the liver and other tissues of experimental animals (Lancaster, Jenkins and Philp, 1961; Sargeant, Sheridan, O'Kelly and Carnaghan, 1961; Schoental, 1963; Feuell, 1966; Wogan, 1966; Rees, 1966; Schoental, 1967; and others). Zuckerman and Fulton (1966) and Zuckerman, Tsiquaye and Fulton (1967) demonstrated the cytotoxicity of aflatoxin B_1 on human embryo liver cells in tissue culture. In the present paper, the morphology of the cells in such cultures and the results of further investigations of the effects of aflatoxins B_1, G_1 and G_2 on these cells are described. A preliminary report of some of these observations on the site of action of aflatoxin B_1 has already been published (Zuckerman, Rees, Inman and Petts, 1967).

MATERIALS AND METHODS

Cell cultures.—Livers were obtained by the Tissue Bank of the Royal Marsden Hospital from normal human embryos after abdominal hysterotomy of women free from infection. The age of the embryos varied from 8–22 weeks. The method of culturing human liver by implantation of liver cells after disaggregation by trypsinization has been described elsewhere (Zuckerman *et al.*, 1967). The tissue cultures were placed in a sealed desiccator with about 2 per cent added CO_2 and incubated at 35°. Within 48 hr. of incubation the implant assumed the appearance of normal hepatic tissue, with most of the cells arranged in a monolayer (Fig. 1a).

The toxins.—These were separated as described by Clifford and Rees (1967). Purified crystalline fractions of aflatoxin B_1, G_1 and G_2, which were kept in the dark at 4° until required, were dissolved in dimethyl formamide (0·04 ml. of the diluent for each 500 μg. of the toxin), diluted immediately in the growth medium and then assayed in the liver tissue culture preparations. A range of doses in steps of 1 and 2 parts per million of the toxin was used to determine the 50 per cent end point. The cultures were floated on the growth medium with and without aflatoxin and with and without the diluent (dimethyl formamide). The preparations were examined, at varying intervals, by fluorescence microscopy after staining with 1/1000 acridine orange, and by phase-contrast microscopy. Purified aflatoxin B_1 was used for the autoradiographic studies. It was dissolved in dimethyl formamide and diluted to give a final concentration of 2 parts per million (2 μg./ml.) in the culture growth medium. The potency of this batch of aflatoxin for human embryo liver cells was established previously by assaying in liver tissue cultures (Zuckerman and Fulton, 1966).

Radioactive labelling.—The radioactive materials used were [^{3}H]-uridine (G) (sp. activity 2·64 curies/mM), 6-[^{3}H]-thymidine (sp. activity 5·0 curies/mM), 4,5-[^{3}H]-lysine (sp. activity 302 mc/mM), [^{3}H]-L-3-phenylalanine (G) (sp. activity 750 mc/mM) and [^{3}H]-L-leucine (G) (sp. activity 200 mc/mM). These were supplied by the Radiochemical Centre, Amersham,

Bucks. Liver cell cultures were exposed to tritiated uridine, using a concentration of 1·0 μc./ml. in growth medium, for 1 hr. In other experiments tritiated thymidine was diluted in the growth medium to give a final concentration of 0·5 μc./ml., and the cells were exposed to this for 1 hr. Labelling of cells with tritiated leucine, lysine or phenylalanine was effected by exposing the cultures to final concentrations of 0·5 μc./ml. of the appropriate amino-acid.

Preparation of cells for autoradiography.—After labelling, the cells were washed with fresh growth medium and fixed in 9 : 1 methyl-acetic mixture for 10 min. Cultures were mounted on microslides treated with 1 per cent gum acacia with the cell surface uppermost; these were then dried in an incubator at 37° for 1 hr. The slides were dipped in a 1 : 5 dilution of Ilford K5 Nuclear emulsion, dried in air overnight at an angle of 60° and subsequently exposed for a suitable time at 4° before development. The preparations were developed in Kodak D.19b at 16° for 5 min., immersed in 3 per cent acetic acid for 10 sec., fixed in Ilford Hypam solution (1 : 4) for 2 min. and washed in running water for 10 min. They were then removed from the slides, stained lightly with haematoxylin and eosin, cleared briefly in xylene and remounted in DePeX.

RESULTS

Cell types

Three types of cell were seen in the cultures of human embryo liver (Fig. 2). The morphology of these cell types, as observed in the light microscope after staining with haematoxylin and eosin was as follows:

Type 1.—Cells of this type were of 2 groups showing slightly different morphology. The first of these, Type 1a, consisted of round or oval cells which were usually seen in the centre of fairly large, closely packed groups of cells but were occasionally present in smaller groups of 2 or 3. They were rarely seen singly. The cell diameters measured from 11–25 μ. The cytoplasm was strongly eosinophilic, granular and without obvious vesiculation. Nuclei were round or oval, the diameters being within the range 6–11 μ. Chromatin was irregularly dense and much more deeply stained than in cells of type 2. Nucleoli were small but deeply stained and often appeared in double or treble forms. In general the morphology of these cells resembled that of hepatocytes. Cells of the second subgroup of Type 1 (Type 1b) were also usually found in large groups of cells, sometimes in the centre of the clumps but more commonly at the periphery. The cell diameters measured from 17–25 μ. The cytoplasm was faintly and irregularly eosinophilic, usually vacuolated and refractile. The nuclei were round, regular and their diameter measured between 9·5–13 μ. The chromatin was less densely stained than in cells of Type 1a, and the nucleoli were paler and more diffuse.

Type 2.—These cells were seen either at the periphery of groups of cells, or singly. The cell outline was irregular, and the diameter of the cells measured from 29–52 μ. The cytoplasm stained faintly grey-blue, and was highly vacuolated and refractile. The diameters of the nuclei measured from 14–19 μ, and the chromatin was loosely packed and very pale-staining. The nucleoli were much paler and more diffuse than in Type 1 cells.

Type 3.—These were spindle-shaped cells. The transverse diameters measured from 11–15 μ whereas the longitudinal diameters were occasionally as great as 70 μ. The cytoplasm was virtually unstained by the technique used. The nuclei were also elongated, having a transverse diameter of from 5–7 μ and a longitudinal diameter of from 15–19 μ. The chromatin in many of these cells was very lightly stained but in occasional cells it was moderately dense. These cells had the morphological characteristics of fibroblasts.

Cytotoxic effects of affatoxin

The cytotoxic effects of the 3 different aflatoxins on the hepatocytes was essentially similar. The earliest changes, as judged by fluorescence microscopy, were observed after exposure for a few hr. to a high concentration of the toxin (10 p.p. million of B_1, 12 p.p. million of G_1 and 30 p.p. million of G_2). These changes consisted of an alteration in the normal granular appearance of cytoplasmic RNA, with green coloration of small areas of the cytoplasm, and loss of definition of the nucleoli. After continuous exposure for about 16 hr. very marked cytopathic changes were observed. The overall dimensions of the hepatic cells were reduced and the cells " rounded up ". There was complete reversal of the normal fluorescence of the cells: the cytoplasm lost the orange fluorescence (RNA), became opaque and fluoresced deep green. In the nuclei of the cells the nucleoli could no longer be identified and there was apparent loss of chromatin material which usually stains green (DNA), but now acquired a deep orange coloration (Fig. 1b).

For the titration of the 3 fractions of aflatoxin 196 cultures were used. The LD_{50} was 1 p.p. million for aflatoxin B_1, 5 p.p. million for aflatoxin G_1 and 16 p.p. million for aflatoxin G_2. The specificity of the cytotoxic action of aflatoxin on the hepatocytes was illustrated by the fact that the spindle-shaped cells (fibroblasts) were unaffected by the toxin (Fig. 1c). Exposure of the cultures to concentrations of G_1 and G_2 fractions lower than those inducing lethal changes caused no apparent immediate alterations in the nuclei of the hepatocytes, and only transient changes were observed in their cytoplasm. The cytological changes, when examined by fluorescence microscopy, were quite characteristic and comprised what may be best described as a green discoloration of a large portion of the cytoplasmic RNA following aflatoxin G_2 (Fig. 1d) and halo-formation following aflatoxin G_1 (Fig. 1e). On further cultivation the parenchymal cells remained alive for up to 23 days.

Effects of aflatoxin B_1 on the incorporation of radioactive compounds

The incorporation of uridine by liver cells in culture was assessed by counting the number of positive cells in autoradiographs; the results are shown in Table I. All the cell types incorporated uridine (Fig. 3). There was a marked difference in the proportion of uridine-labelled cells within cell types 1a and 1b, namely 35 per cent and 93 per cent respectively. The addition of aflatoxin B_1 in low

TABLE I.—*The Effect of Aflatoxin B_1 on the Incorporation of Uridine by the Various Cell Types Present in Human Embryo Liver Cultures*

	Control cultures		Cultures treated with aflatoxin		
Cell type	Number of cells counted	Number and percentage of positive cells	Number of cells counted	Number and percentage of positive cells	Reduction in positive cells (per cent)
a 1	1050	350 (33·3 per cent)	1000	9 (0·93per cent)	97·3
b 2	1000	914 (91·4 per cent)	400	23 (5·7 per cent)	94
	150	143 (95·3 per cent)	200	154 (77 per cent)	19·2
3	1050	1004 (95·6 per cent)	800	728 (91 per cent)	4·8

concentration (2 p.p. million) to the cultures reduced the numbers of positive cells to a different extent for the 3 cell types. The toxin strongly inhibited the uridine incorporation in cell type 1 but scarcely affected that of cell type 3.

TABLE II.—*The Effect of Aflatoxin B_1 on the Incorporation of Thymidine by the Various Cell Types Present in Human Embryo Liver Cultures*

	Control cultures		Cultures treated with aflatoxin		
Cell type	Number of cells counted	Number and percentage of positive cells	Number of cells counted	Number and percentage of positive cells	Reduction in positive cells (per cent)
a 1	1000	30 (3 per cent)	1000	12 (1·2 per cent)	60
b	1000	17 (1·7 per cent)	1000	15 (1·5 per cent)	12
2	1000	197 (19·7 per cent)	1000	65 (6·5 per cent)	67
3	1000	64 (6·4 per cent)	1000	42 (4·2 per cent)	34·4

The results shown in Table II indicate that, although a proportion of all types of cells incorporated thymidine, the proportion of positive cells was very much lower than had been observed in the case of uridine incorporation. The percentage of type 2 cells incorporating thymidine (Fig. 4) was considerably greater than that of either types 1 or 3. Aflatoxin reduced the percentage of positive cells of all cell types, producing the greatest reduction in positivity in cell type 2.

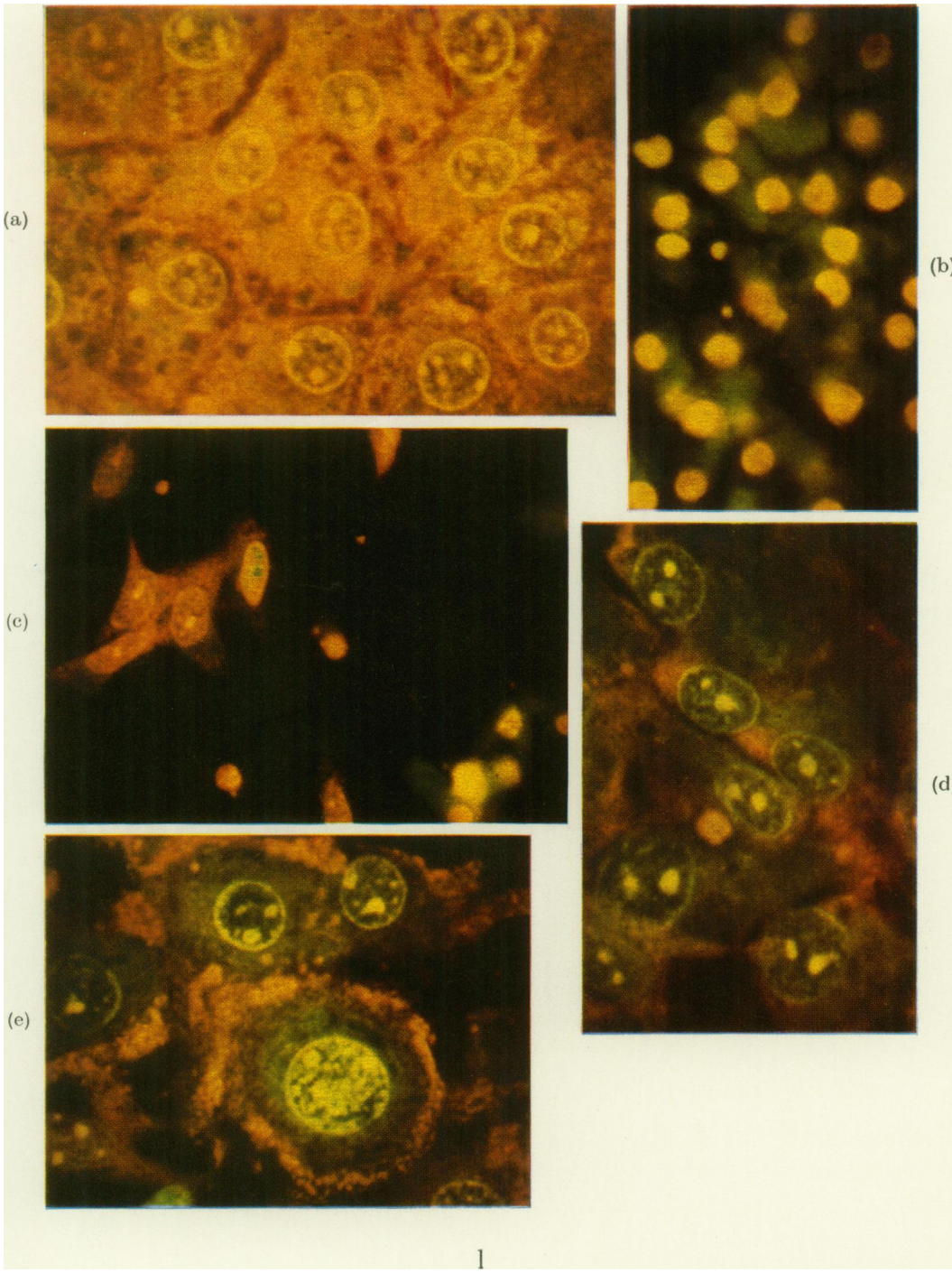
EXPLANATION OF PLATES

FIG. 1.—Fluorescent micrographs of cultured human embryo liver cell preparations stained with 1 : 1000 acridine orange.
(a) a confluent layer of normal human embryo liver cells after 48 hr. in culture.
(b) after exposure to aflatoxin B_1 (10 p.p. million) for 16 hr. The cells have rounded up, the cytoplasm is stained opaque green and the nuclei fluoresce a deep orange colour.
(c) after exposure to aflatoxin G_1 (12 p.p. million). The hepatocytes show the same characteristic cytotoxic changes as in (b) but the spindle-shaped cells (fibroblasts) are unaffected by the toxin.
(d) after continuous exposure to aflatoxin G_2 (10 p.p. million) for 48 hr. Note the green discolouration of large portions of the cytoplasm.
(e) after exposure to a sub-lethal concentration of aflatoxin G_1 (2 p.p. million) for 30 hr. Note the characteristic " halo " formation in the cytoplasm.
Magnification: ×900 throughout.

FIG. 2.—Phase contrast photomicrograph of normal human embryo liver cells after 48 hr. in culture. Examples of the cell types described in the text are indicated on the micrograph. ×500.

FIG. 3.—Phase contrast photomicrographs of an autoradiograph of normal human embryo liver cells after 48 hr. in culture. The cells had been exposed to tritiated uridine for 1 hr. before fixation, as described in the text.
(a) type 1b cells
(b) type 2 cell
(c) type 3 cells
The positivity is almost completely confined to the nuclei of the cells, indicating that migration of newly synthesized RNA into the cytoplasm had not commenced before fixation. ×1000.

FIG. 4.—Phase contrast photomicrograph of an autoradiograph of normal human embryo liver cells after 48 hr. in culture. The cells had been exposed to tritiated thymidine for one hour before fixation, as described in the text. Several type 2 cells are included, only one of which is in DNA synthesis. ×1000.



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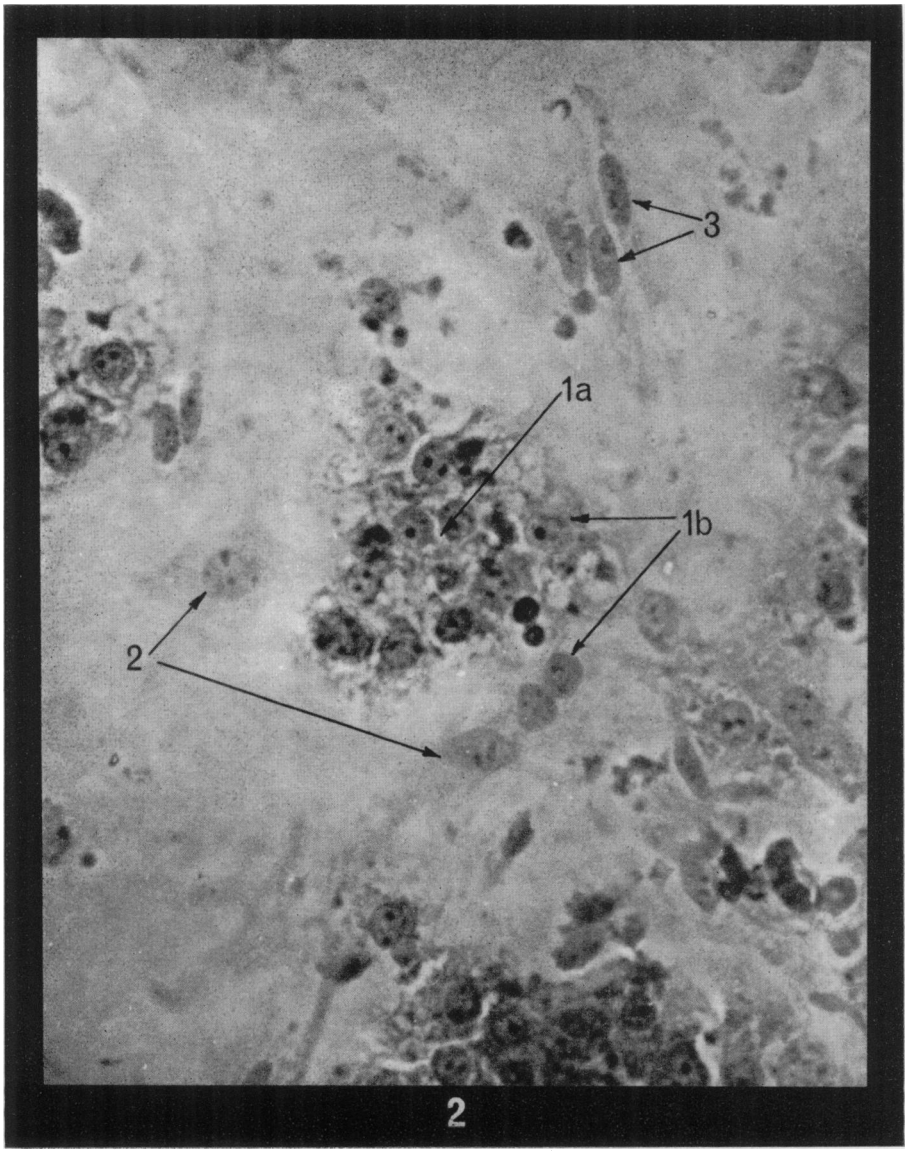
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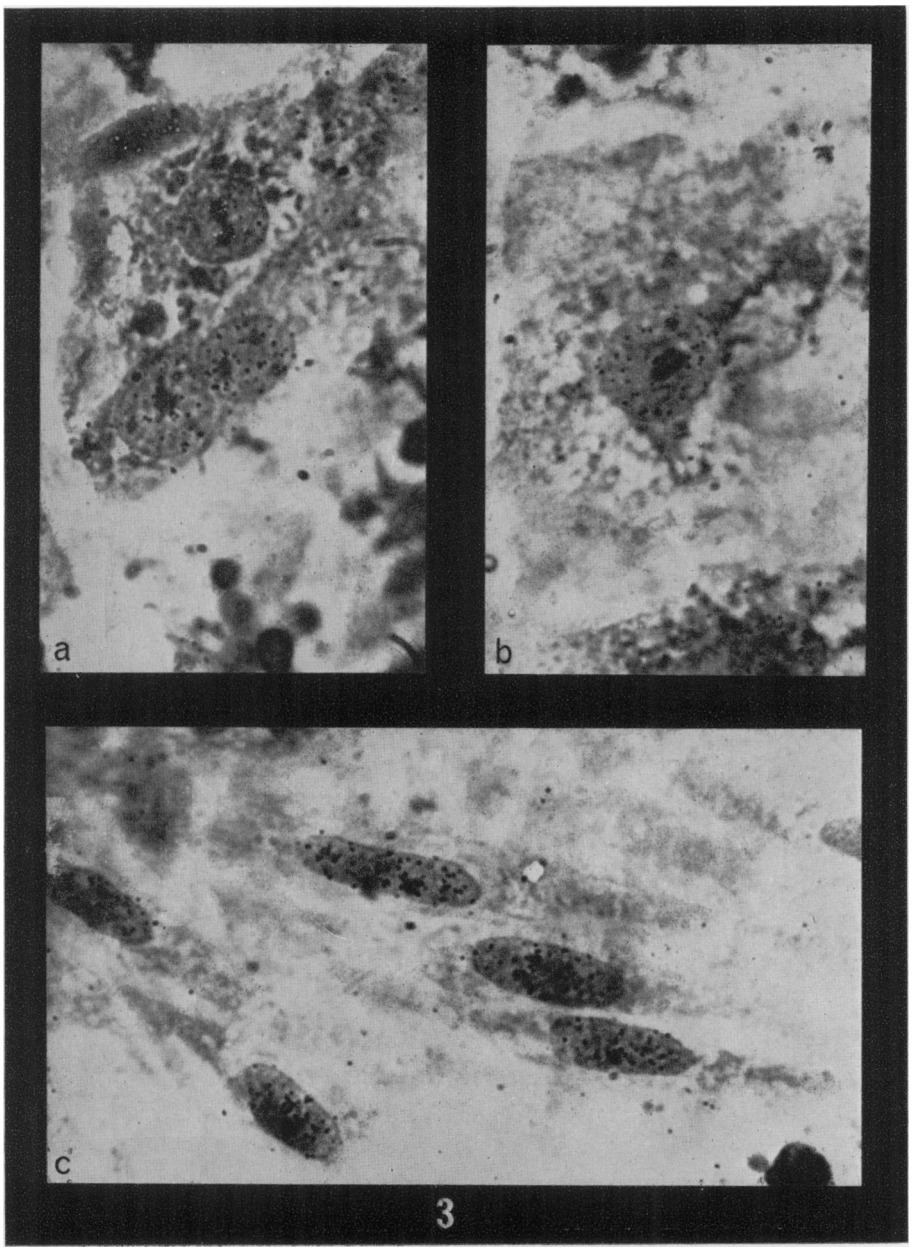

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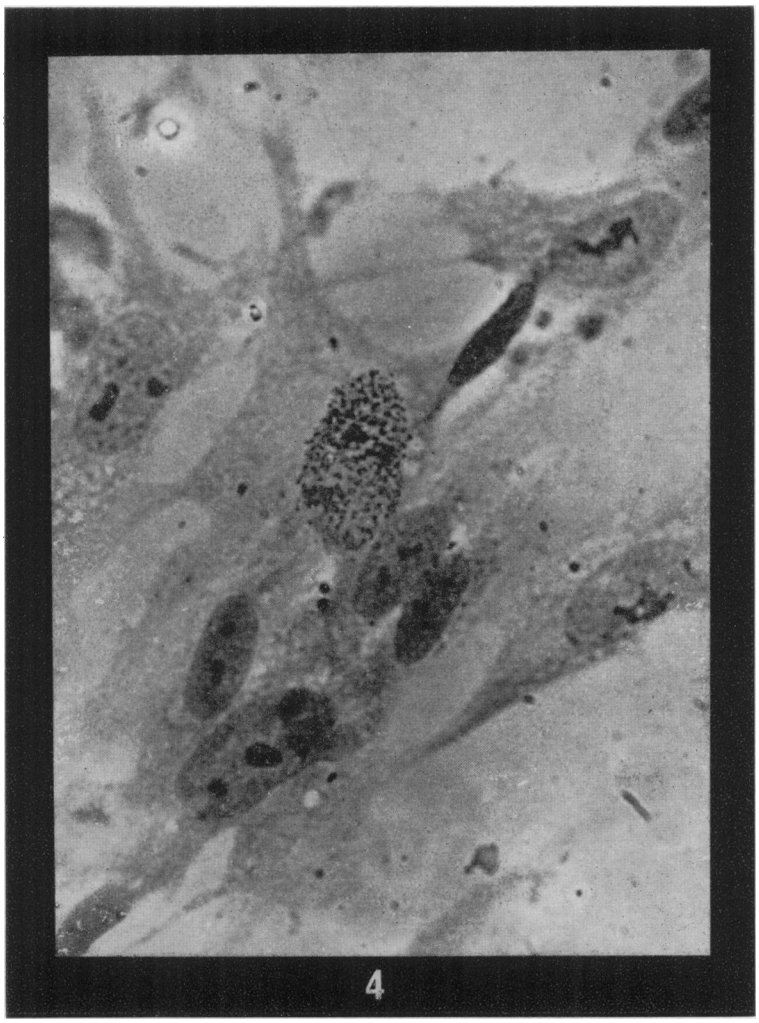

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Preliminary experiments in which labelled amino-acids were added to the growth medium of the liver cell cultures, showed that the cells readily incorporated tritiated leucine, lysine and phenylalanine. Pretreatment of the cells with aflatoxin B_1 for 1 hr. appeared to lead to a reduction in the ability of the cells to incorporate leucine. The effects of the toxin on the cells capacity for lysine and phenylalanine incorporation has not yet been investigated.

DISCUSSION

The results given above show that human embryonic liver cells in culture are able to incorporate thymidine, uridine and various amino acids when these are added to the culture medium. This may be interpreted as evidence that the cells are capable of synthesizing DNA, RNA and protein under the conditions employed in the cultures. The incorporation of thymidine by an appreciable number of cells showed that these were in the S-period of their cycle at the time when they were presented with thymidine. The percentage of cells synthesizing DNA was about twice that which may be calculated for rat hepatocytes *in vivo* on the basis of observed mitotic indices (Bucha, 1963).

A number of cell types were present in the liver cultures. Disregarding those cells which clearly belonged to the haemopoietic series, 3 cell types were distinguished. Type 1 cells may be regarded as hepatocytes on the basis of their morphology. Two subgroups of cells were observed within this type and designated 1a and 1b; the appearance of type 1b suggested that these cells may have been at an early stage of transformation or dedifferentiation. The morphology of type 2 cells left little doubt that they were relatively undifferentiated. The staining characteristics of their nuclei suggested that there was a high degree of derepression of the chromatin. Furthermore, the staining properties of the cytoplasm were indicative of a high RNA content suggesting that they were actively engaged in protein synthesis. The significance of cell type 2 is not yet clear; it may be that these cells were the result of either transformation or dedifferentiation of type 1 cells. On the other hand it is possible that they represented partially differentiated cells which were present in the original liver sample. If so, this may be a reflection of the fact that the cell samples were taken from the livers of embryos between the first and second trimester of pregnancy.

Type 3 cells were morphologically identical to fibroblasts. Whether these resulted from dedifferentiation in the culture is not certain, but this seems unlikely in view of the fact that the cells studied during these investigations had all been in culture 48 hr. or less. It is most probable, therefore, that type 3 cells were present in the original embryonic liver as connective tissue cells and were transferred as such to the culture.

The validity of the morphological classification of the three cell types was supported by the autoradiographic evidence of the incorporation of nucleic acid precursors. These results suggest that only 30 per cent of type 1a cells were synthesizing RNA. It must be borne in mind, however, that this may reflect the sensitivity of the autoradiographic method; apparently negative cells may have been synthesizing RNA at too low a rate to be detected. On the other hand, 90 per cent of the cells in type 1b were demonstrably positive and these cells must, therefore, have been synthesizing RNA more actively. In this respect, type 1b cells were comparable with those of type 2. The proportion of type 2 cells in DNA synthesis was 10 times that of type 1 cells, and 3 times that of type 3 cells.

Further evidence of the functional differences between the three cell types was given by their response to the aflatoxins. Fluorescence microscopy did not show any differences between the response of type 1 cells and that of type 2 cells to aflatoxin. The autoradiographic evidence, however, showed an almost complete inhibition of detectable RNA synthesis in type 1 cells after 3 hr. exposure to aflatoxin B_1, whereas some 80 per cent of type 2 cells continued to synthesize RNA under the same conditions. This marked difference between the effects of aflatoxin on cells of type 1 and on those of type 2 shown by autoradiography was not detected by fluorescence microscopy following acridine orange staining. This probably reflects the processes which were revealed by these techniques; autoradiography gave a measure of nucleic acid synthesis, whereas fluorescence microscopy revealed the nucleic acid content of the cells. The specificity of the cytotoxic action of aflatoxin on the hepatocytes is illustrated by the striking observation that cells of type 3 (fibroblasts) were unaffected by the toxin as measured by either fluorescence microscopy or autoradiography, Clifford and Rees (1966, 1967) found that aflatoxin entered the nucleus of the rat liver cell, interacted with the DNA and thereby inhibited its transcription by the DNA and RNA polymerases; this resulted in an inhibition of the synthesis of DNA and messenger-RNA. The inhibition of messenger-RNA synthesis in turn resulted in an inhibition of protein synthesis. In the present studies aflatoxin inhibted RNA, DNA and protein synthesis in the human cells in culture and it is reasonable to suppose, therefore, that the mechanism of action of the toxin is similar to that in the rat.

The different effects of aflatoxin on nucleic acid synthesis in the different cell types may be due to a variation in the ability of the toxin to combine with DNA, or the relative permeabilities of the cells to the toxin, or the rates of metabolic breakdown of the toxin within the cells. The last of these possibilities seems unlikely in view of the fact that the synthesis of RNA by type 2 cells was relatively unaffected by the toxin as compared with their DNA synthesis. These problems require further elucidation.

The order of toxicity of aflatoxin B_1 and G_1 in human liver cell cultures was similar to the order of carcinogenicity observed by Dickens and Jones (1964, 1965) who produced local sarcomas in rats by the injection of aflatoxins. They found that the induction period of the tumours with B_1 was approximately a third of that with G_1. The present investigation on human liver cells, has, however, been limited to short term cultures and these were not suitable for the study of cell transformation over a period of time.

SUMMARY

The morphology of the cell types present in cultures of human embryo liver are described. Three principal cell types were present, apart from cells belonging to the haemopoietic series.

All 3 cell types were found to synthesize DNA and RNA, with incorporation of radioactive labelled precursors. The proportions of cells synthesizing nucleic acids differed in the 3 cell types. There was also evidence of protein synthesis in the hepatocytes.

The cytotoxic effects of 3 aflatoxins on the liver cells are described.

Aflatoxin B_1 in a concentration of 2 p.p. million (2 μg./ml.) inhibited DNA and RNA synthesis in the hepatocytes. These results are discussed in relation to previous observations in experimental animals.

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