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CHANGES IN MORPHOLOGY OF CELL CULTURES AFTER TREATMENT WITH AFLATOXIN AND OCHRATOXIN*

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Interest in the effects of mycotoxins in animals and man has developed in the last decade largely owing to the dramatic biological properties of aflatoxin. Apart from the liver necrosis produced after acute poisoning,[1] aflatoxin is one of the most potent hepatocarcinogens yet described. It has been postulated that aflatoxin or other mycotoxins may be involved in the aetiology of malignant hepatoma in man.[2-4] Recent studies of aflatoxin have revealed some of its biochemical effects in experimental animals,[5-7] and observations on human liver cell cultures indicate that the same biochemical lesions occur in human liver.[8]

Other mycotoxins[9,10] have been purified from moulds which were isolated in a systematic search for toxic fungi on South African crops. Ochratoxin A, a substituted isocoumarin isolated from *Aspergillus ochraceus*,[9] has an LD_{50} in rats of 20 mg./kg. and produces a pronounced tubular necrosis of the kidney and a disturbance in glycogen metabolism in the liver.[11,12]

Monkey kidney epithelial cell cultures have been exposed to aflatoxin and ochratoxin to determine whether any specific morphological effects are produced which give an indication of the mode of action of the toxins.

METHODS

Kidney epithelial cells of the African green monkey *Cercopithecus aethiops pygerythrus* were cultured as mono-layers on glass coverslips in roller tubes. The culture medium was Hank's basic salt solution modified by the addition of 0·5% lactalbumin hydrolysate, 5% horse serum (inactivated at 56°C for 20 minutes), 0·1 mg. streptomycin sulphate/ml. medium, 0·05 mg. neomycin sulphate/ml. medium and 100 units penicillin G/ml. medium. The cultures were kept stationary for the first 5 - 7 days, when the medium was renewed and the tubes were placed in roller drums. The medium was renewed every 2 - 3 days until the monolayer was confluent.

The cultures were then exposed to the mycotoxins for 24 and 48 hours, fixed in Bouin's fixative, stained with Ehrlich's haematoxylin and eosin, dehydrated in alcohol and mounted with Canada balsam.

Aflatoxin was added to the culture medium as a solution in dimethyl sulphoxide (DMSO). Various quantities of aflatoxin were dissolved in DMSO so that the required concentration of aflatoxin in the culture medium was obtained by the addition of 0·1% DMSO.

The sample of aflatoxin used was crystalline and contained 92% of aflatoxin in the proportion of 72·5% B_1, 24% B_2, 2% G_1 and 1·5% G_2.

Ochratoxin A was dissolved directly in the culture medium. The sample used was crystalline and in the form of the sodium salt.

Control cultures were incubated in fresh medium or in the medium containing 0·1% DMSO, for 24 - 48 hours. Each experiment, which consisted of adding a known concentration of mycotoxin to 3 tubes, was repeated 3 times or more. Cells showing the various changes described were counted on parts of the coverslip which had a single layer of cells. All counts are expressed as percentages of the total number of cells counted, which was between 1,000 and 3,000 per coverslip.

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RESULTS

Normal Cell Culture

The cultures had formed a confluent layer of cells on the coverslip when toxins were added, with some areas having 2 or more layers. The majority of cells and nuclei were of uniform size and shape (Fig. 1(*a*)). Between 1 and 4 fairly prominent nucleoli of uneven shape were present in the nuclei. A mitotic rate of more than 1·45% was usually seen.

Cultures to which DMSO alone was added had similar characteristics except that a slight increase in mitosis was seen.

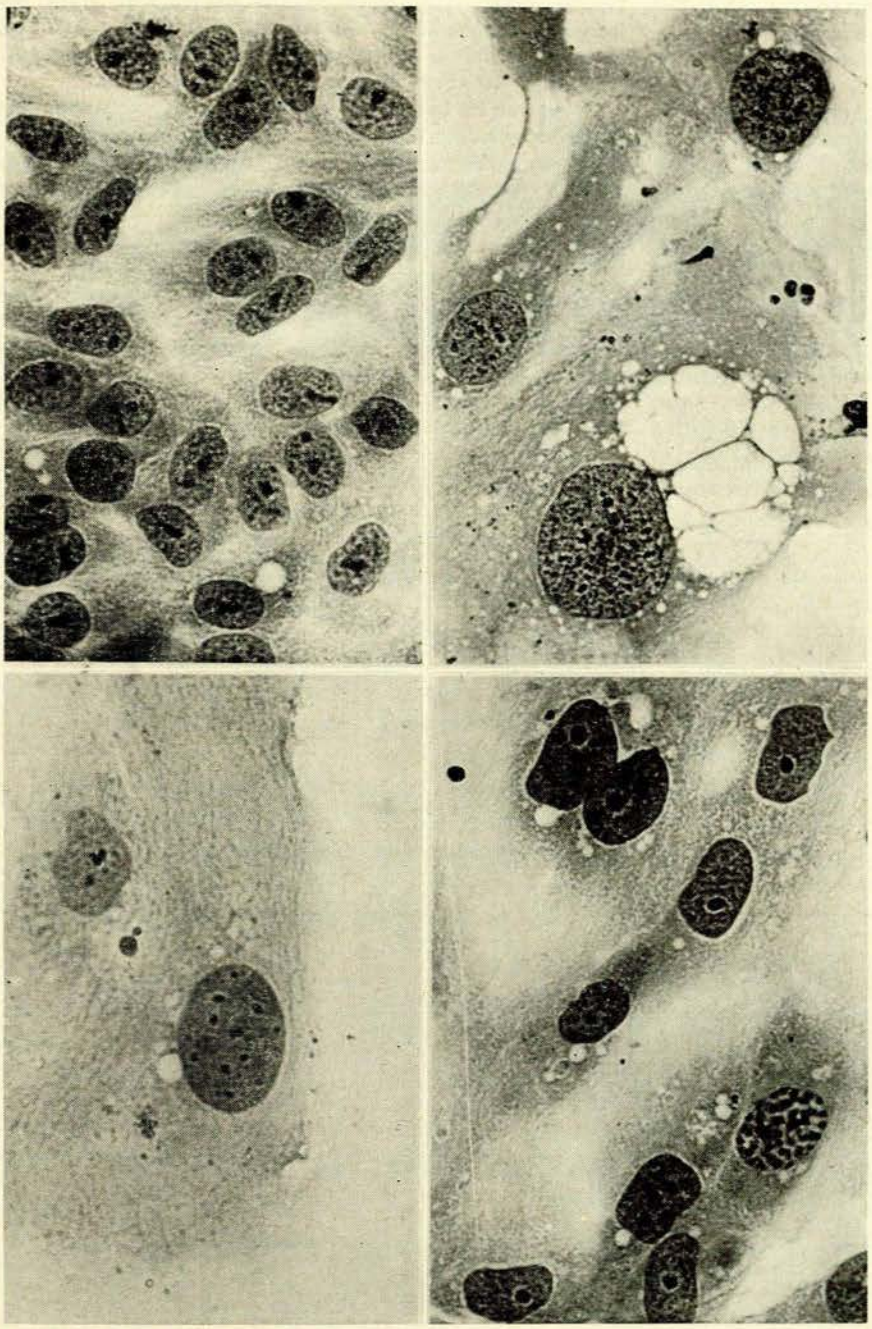
Aflatoxin

At 0·025 - 0·25 μg./ml. for 24 hours. There was a decrease in the cell count after 24 hours and in the percentage of mitotic figures (Table I). At concentrations between 0·125 and 0·25 μg./ml. there was a variation in size and structure of the nucleoli. The nucleoli seemed to be separated or fractured into 2 or more parts. These 'fractured' nucleoli were small and inconspicuous. Some were basophilic and some acidophilic. Degenerative changes were observed, which included karyorrhexis, pycnosis, increased cytoplasmic vacuolation and an increase in the amount of cell debris (Table I).

At 0·025 - 0·25 μg./ml. for 48 hours. After an exposure to aflatoxin for 48 hours there was a further decrease in the percentage of mitotic figures for concentrations of aflatoxin up to 0·18 μg./ml. No mitosis was observed at higher concentrations after 48 hours of exposure (Table I). Nuclei having more numerous but smaller nucleoli increased considerably. Many cells with extensive vacuolation in cytoplasm were observed, while the number of cells per field decreased (Table I).

At 0·5 - 1·0 μg./ml. for 24 hours. At 24 hours there were no mitotic figures in the lower concentrations. Pycnosis and karyorrhexis were increased, as were cells with cytoplasmic vacuolation (Fig. 1(*b*)). More nuclei with folds were observed and nuclei with fragmented nucleoli increased considerably (Table I, Fig. 1(*b*)).

At 0·5 - 1·0 μg./ml. for 48 hours. No mitotic figures were seen at 48 hours. The nuclei, in addition to having a number of smaller nucleoli (most of which were eosinophilic) showed a loss of chromatin material. A steady increase in the number of nuclei with folds was observed as the dose of aflatoxin increased (Table I). Most nuclei at this stage had smaller more numerous eosinophilic nucleoli.



Above: Fig. 1(a) and 1(b)

Below: Fig. 1(c) and 1(d)

Fig. 1. Culture of green monkey epithelial cells (× 640) stained with haematoxylin and eosin. *1(a).* Untreated cell cultures. *1(b).* Cell cultures after 24 hours' exposure to aflatoxin (0·5 μg./ml.), showing cytoplasmic vacuolation and fragmented nucleoli. *1(c).* Cell cultures after 24 hours' exposure to aflatoxin (5·0 μg./ml.). Nuclei showing loss of chromatin material and many small nucleoli or inclusion bodies. *1(d).* After 24 hours' exposure to ochratoxin (1·6 μg./ml.), showing cell with condensed chromatin resembling the prophase of normal mitosis. Nucleoli appear more rounded than those of control culture, with a lighter-staining area (halo) surrounding most of them.

The proportion of cells showing extensive cytoplasmic vacuolation, pycnosis and karyorrhexis increased (Table I), although only a few cells per field remained.

At 5·0 μg./ml. for 24 hours. No mitotic figures were seen at this concentration. The loss of nuclear chromatin was marked at 24 hours, giving the nuclei a 'ghost-like' appearance with small (pin-point) nucleoli (Fig. 1(*c*)) occurring in most nuclei (Table I). Many nuclei had folds giving them an irregular appearance. Degenerating cells (nuclei showing pycnosis and karyorrhexis) increased (Table I) with a similar increase in cell debris.

At 5·0 μg./ml. for 48 hours. At 48 hours the most striking change was the decrease in cell numbers. Karyorrhexis and pycnosis were noticeable, and of the remaining cells many showed rounding of the cell with vacuolation. 'Ghost' cells, with nuclei containing very little chromatin but with numerous small nucleoli, predominated (Fig. 1(*c*), Table I).

At 10 μg./ml. for 24 hours. Most of the degenerative changes seen in the lower concentration were more frequently encountered.

At 10 μg./ml. for 48 hours. Degenerative changes predominated, with a marked increase in pycnosis and karyorrhexis. Most of the remaining cells had fragmented nucleoli.

Ochratoxin

At 0·1 - 0·6 μg./ml. The cells and nuclei appeared normal after 24 hours of exposure to the toxin. There was, however, a decrease in mitotic figures. After 48 hours this decrease in mitotic figures was more marked.

At 0·8-3·2 μg./ml. Striking changes in the appearance of the nuclei of many cells were noticed at 24 hours. The chromatin of some cells was condensed, resembling the prophase of normal mitosis (Fig. 1 (*d*)). It was not certain that this was a true prophasic change, since it occurred in small, fragmented nuclei which do not normally undergo mitosis (Fig. 2 (a) and (b)). These cells with fragmented nuclei appeared to have been arrested in

a 'pseudoprophasic' stage before fragmentation occurred.

The chromosomes of some other cells which were in mitosis were considerably shortened and the cells appeared to be blocked in metaphase (Fig. 2(*b*)). The cytoplasm of these cells was strongly eosinophilic. No normal mitotic figures were seen in treated cultures in most experiments, although in some experiments a few normal mitotic figures were seen at 0·8 μg./ml. In all experiments, however, at a dose of 1·6 μg./ml. all mitotic figures appeared abnormal (Table II).

A high percentage of the remaining cells had nuclei which contained one or two distinct nucleoli surrounded by a halo (Figs. 1(*d*) and 2(*b*), Table II).

An increase in pycnosis and karyorrhexis was noticed.

After 48 hours' exposure, cells showing the shortened chromosomes (seen at 24 hours) were rare, and in some experiments no mitotic figures were found. Degenerative changes in nuclei (pycnosis, karyorrhexis) and cytoplasm (vacuolation) were increased and nuclei with folds and irregular shapes were also common (Fig. 2(*a*) and (*c*)). In many cultures an increase in multiple nucleated cells was observed (Fig. 2(*a*)).

Other nuclei had large prominent nucleoli surrounded by a distinct halo (Fig. 2(*c*)).

In one experiment with a dose of 1·6 μg./ml. no mitotic figures were seen at 67 hours, but otherwise the changes were similar to those seen at 48 hours, although some nucleoli appeared more condensed and larger with a very distinct halo.

At 6·4 - 12·8 μg./ml. After 24 hours' exposure there was an increase in most abnormal and degenerative changes, except that the cell population, mitotic figures and number of fragmented nuclei decreased (Table II). Some nuclei with clumped chromatin were observed, but they did not resemble prophase nuclei.

The changes seen at 48 hours were similar but more

TABLE I. CHANGES IN CELL MORPHOLOGY EXPRESSED AS A PERCENTAGE OF TOTAL CELLS COUNTED AFTER EXPOSURE TO AFLATOXIN FOR 24 OR 48 HRS (1,000 CELLS WERE COUNTED FOR EACH CONCENTRATION EXCEPT THOSE MARKED WITH AN ASTERISK)

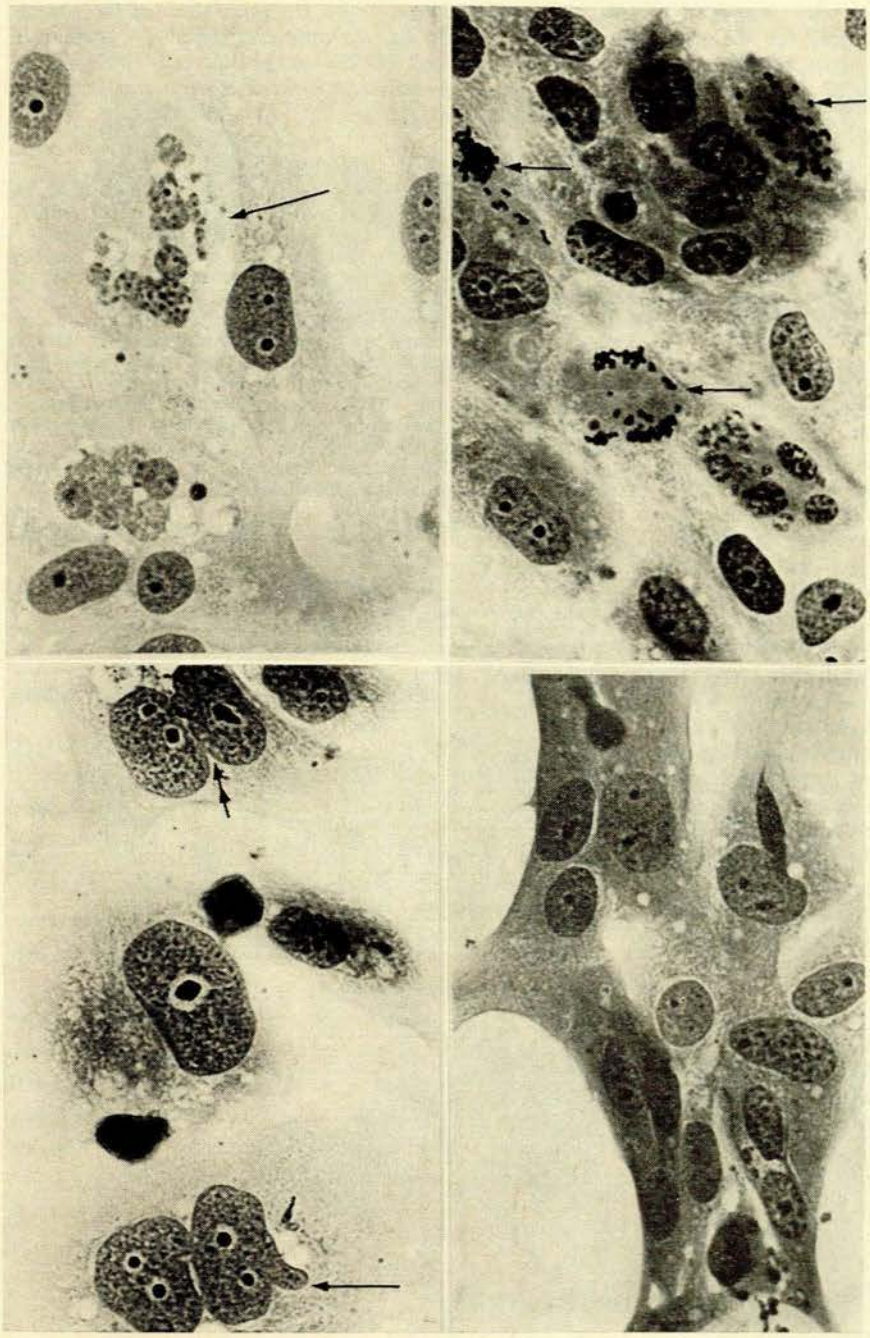
Aflatoxin μg./ml.	*Cells*	*Fields*	*Nuclei*							*Cytoplasm vac.*	*Nucleoli frag.*
			Pycn.	*Karyo.*	*Folds*	*Double*	*Multiple*	*Large*	*Mitotic*		
After 24 hours											
0·0	31	32	0·6	0·6	0·7	1·1	0·3	0·8	2·1	3·8	0·0
0·25	14	71	0·6	2·5	0·5	1·5	0·0	0·9	0·03	12·8	19·2
0·50	11	91	2·1	7·4	1·4	1·6	0·2	0·9	0·0	13·6	43·2
1·0	15	66	2·5	6·9	4·9	2·8	0·7	1·4	0·0	10·4	61·4
5·0	5	200	2·0	6·4	6·3	1·7	0·1	1·0	0·0	11·4	80·5
10·0	4	250	4·9	5·3	4·9	2·3	0·3	1·5	0·0	16·0	82·0
After 48 hours											
0·0	26	38	0·8	0·2	0·9	1·3	0·2	2·1	1·6	7·5	0·0
0·25	7	125	1·1	3·0	3·2	2·9	0·3	2·4	0·0	29·0	67·3
0·50	3	333	3·0	7·2	5·0	3·2	0·2	2·0	0·0	35·0	78·4
1·0*	2	240	5·0	12·0	6·0	4·4	0·0	2·6	0·0	30·4	73·0
5·0*	0·7	714	9·0	17·0	6·0	6·0	0·1	4·0	0·0	25·0	62·0
10·0*	0·5	1,000	22·0	33·0	7·0	2·0	0·0	1·0	0·0	14·0	39·0

Pycn. = pycnosis; karyo. = karyorrhexis; cytoplasmic vacuolation = cells with more than 3 distinct vacuoles in the cytoplasm or with 1 or 2 vacuoles with a diameter greater than approximately 5μ; fragmented nucleoli = small irregular basophilic and acidophilic bodies in the nucleus, which may be fragmented nucleoli. In some nuclei from cells exposed to a lower concentration of aflatoxin the acidophilic and basophilic part of the nucleolus were not separate but clearly distinguishable. Similar changes have been described in liver nuclei.[18]

TABLE II. CHANGES IN CELL MORPHOLOGY EXPRESSED AS A PERCENTAGE OF TOTAL CELLS COUNTED AFTER 24 AND 48 HOURS' EXPOSURE TO OCHRATOXIN (1,000 CELLS COUNTED FOR EACH CONCENTRATION)

Ochratoxin	*Cells/field*	*Fields*	*Nuclei*										*Nucleoli with halo*	*Vacuol. cyto.*
			Pycn.	*Karyo.*	*Folds*	*Double*	*Multiple*	*Large*	*Mitotic*	*Multiple clumped*	*Prophase*	*Metaphase*		
After 24 hours														
0·0	31	32	0·6	0·6	0·7	1·1	0·3	0·8	2·1	0·0	0·0	0·0	0·0	3·8
0·8	25	40	0·8	1·0	3·1	1·8	0·6	2·4	0·7	0·8	0·1	0·6	23·7	10·7
1·6	33	30	1·2	1·0	1·1	1·2	1·3	1·7	0·9	0·9	0·2	0·7	36·0	4·6
3·2	25	40	4·0	3·4	2·9	2·4	0·9	1·7	0·4	0·7	0·3	0·1	46·3	12·7
6·4	18	55	4·1	3·9	2·5	1·6	0·1	2·6	0·6	0·1	1·1 *	0·1	39·4	8·9
12·8	13	77	2·1	2·9	1·8	3·0	0·6	2·1	0·3	0·3	1·6 *	0·0	55·2	13·7
25·6	13	77	5·0	4·4	4·7	2·8	0·2	3·6	0·0	0·0	1·2 *	0·0	34·6	9·5
After 48 hours														
0·0	26	39	0·8	0·2	0·2	1·3	0·2	2·1	1·6	0·0	0·0	0·0	2·9	7·5
0·8	20	50	3·8	14·0	12·4	1·9	0·4	3·9	0·0	0·0	0·0	0·0	34·9	11·9
1·6	16	63	1·4	5·7	6·2	3·8	1·7	6·5	0·0	0·0	0·0	0·0	36·4	9·8
3·2	16	63	3·0	11·9	7·9	1·5	0·8	3·9	0·0	0·0	0·0	0·0	52·0	14·9
6·4	17	59	8·9	19·0	11·1	2·3	0·1	4·0	0·0	0·0	0·0	0·0	45·6	14·0
12·8	8	125	6·3	25·9	10·9	2·3	0·2	5·2	0·0	0·0	0·0	0·0	54·0	32·3
25·6	12	84	5·8	22·6	15·8	2·0	0·6	4·4	0·0	0·0	0·0	0·0	44·5	28·5

The terms used are as in Table I, with the addition of the following: Multiple clumped (multiple nuclei with clumped chromatin material) = cells containing more than 2 nuclei which varied in size up to a quarter of the diameter of a normal nucleus (these appeared to be cells in prophase with fragmented nuclei); prophase = nuclei with a typical prophase appearance (other cells containing fragmented nuclei in prophase suggest that these cells may represent a prophasic block); metaphase = cells in metaphase with shortened chromosomes scattered throughout the nucleus, the cytoplasm being more eosinophilic than normal dividing cells; nucleolus with halo = rounded dense-looking nucleolus surrounded by a non-staining area or halo—the nucleolus was larger than normal (up to 4 times the normal diameter), and the halo gave it a prominent appearance.
*Marks cells with clumped chromatin—an early degenerative change consisting of an increase in the chromatin granules in the nucleus (Fig. 2(*d*)).



Above: Fig. 2(a) and 2(b)
Below: Fig. 2(c) and 2(d)

Fig. 2. Cultures of green monkey epithelial cells (× 640) stained with haematoxylin and eosin. *2(a).* Cell culture after 48 hours' exposure to ochratoxin (1·6 μg./ml.), showing two multiple nucleated cells, one of which (arrow) appears to have a fragmented nucleus in prophase. *2(b).* After 24 hours' exposure to ochratoxin (3·2 μg./ml.). Three cells are blocked in metaphase (arrows) and one cell has a fragmented nucleus 'blocked in prophase'. *2(c).* After 48 hours' exposure to ochratoxin (3·2 μg./ml.) more large cells with a very prominent nucleolus and halo are seen. Irregular nuclei (arrow) and folds (double arrow) in nuclei have increased. *2(d).* Cell culture after 24 hours' exposure to ochratoxin (25·6 μg./ml.), showing several nuclei with clumped chromatin granules as well as considerable cell damage.

pronounced than those seen at 24 hours. In particular, nucleoli appeared more prominent with a halo after 48 hours.

At 25·6 μg./ml. No mitotic figures were observed at 24 hours. Most other changes were similar to, but more extensive than, those seen at lower concentrations (Table II), with the exception that nucleoli were not prominent (Fig. 2 (*d*)). Nuclei with clumped chromatin were observed more frequently (Fig. 2(*d*)).

At 48 hours degenerative changes were very extensive.

DISCUSSION

Many of the morphological changes seen in cell cultures exposed to aflatoxin and ochratoxin may be the changes normally seen in dying cells. Pycnosis, karyorrhexis and cytoplasmic vacuolation are seen in normal cultures, and an increase in their incidence reflects a cytotoxic effect of aflatoxin resulting in cell death and a decrease in the number of cells in the culture. These changes, which have been observed before,[14,15] are tabulated here for comparative purposes. Other morphological changes observed are absent in normal cultures and may reflect an alteration in cellular metabolism caused by the aflatoxin or ochratoxin.

Aflatoxin produced a decrease in the number of mitotic figures with complete inhibition of mitosis after 24 hours of exposure to aflatoxin (0·25 μg./ml.). Legator[15,16] observed a similar effect in human embryonic lung cells. Simultaneously there is an increase in the number of enlarged cells ('giant' cells) to a higher percentage than that reported previously.[16]

Probably the most significant change was the decrease in the size of the nucleolus with 'fragmentation' occurring in the vast majority of cells. Zuckerman *et al.*[5] reported 'loss of definition' of the nucleoli in liver cells, and Svoboda *et al.*[13] described the formation of nucleolar 'caps' after aflatoxin administration to rats and monkeys. Electron-microscopical examination of the nucleolar changes in rats and

monkeys revealed that the nucleolus had segregated into granular and fibrillar parts.[13] Actinomycin D also produces segregation of the nucleolar components and nucleolar caps.[17] The biochemical effects of aflatoxin and actinomycin D have certain characteristics in common, such as inhibition of RNA polymerase, messenger RNA production and protein synthesis.[6] Inhibition of protein synthesis,[14] DNA synthesis[15] and RNA synthesis[16] has been observed in various cell cultures. The interference in nuclear DNA and RNA metabolism may well be responsible for the morphological changes observed. Similarly the decrease in chromatin-producing ghost-like nuclei (also observed by Zuckerman *et al.*)[8] may result from these biochemical changes.

Apart from the accentuated degenerative changes similar to those seen after aflatoxin, ochratoxin A produced several unusual morphological changes. There was a marked decrease in the percentage of cells in normal mitosis, but it is interesting that at 24 hours the total number of cells undergoing some form of mitosis remained fairly constant. Thus, as the percentage of mitotic cells decreased, those in pseudoprophase and blocked metaphase increased. The addition of ribonuclease to fibroblast cultures results in cells becoming blocked in 'pseudoprophase',[19] apparently unable to proceed to cell division. Ribonuclease also produces metaphase block in cultures.[20] It seems that the effects of ochratoxin A on mitosis are similar to those produced by ribonuclease. Whether the action of ochratoxin A involves an increase in ribonuclease activity is not known, but recent work has shown that there is a decrease in rat kidney RNA after ochratoxin A administration.[21] This would support the idea that ochratoxin A either stimulates ribonuclease or has an action with characteristics similar to ribonuclease.

Fragmented or multiple nuclei containing the clumped chromatin morphologically similar to that seen in 'pseudoprophase' cells may reflect a toxic action of ochratoxin superimposed on the other effects.

The cells not involved in mitosis after ochratoxin A administration have large rounded nucleoli. Morphological changes in the nucleolus may also be related to alterations in RNA metabolism, and may be a reflection of a similar biochemical effect on non-mitotic cells.

The morphological changes observed after ochratoxin A and aflatoxin treatment of cell cultures differed considerably. Aflatoxin produced fragmentation of the nucleolus which could be due to inhibition of RNA formation. Ochratoxin produced effects on mitotic cells which could result from an entirely different action, namely, an increase in RNA catabolism.

SUMMARY

Aflatoxin and ochratoxin produced specific cytological alterations in African green monkey kidney epithelial cell cultures after 24 and 48 hours of exposure. Aflatoxin produced a decrease in mitosis and fragmentation of the nucleolus, as well as non-specific changes such as cytoplasmic vacuolation and pycnosis or karyorrhexis. Ochratoxin produced enlarged nucleoli surrounded by a non-staining halo at all doses. A decrease in normal mitosis was observed simultaneously with an increase in abnormal forms. Prophase and metaphase blocks were observed at 24 hours, but at 48 hours no mitotic figures were seen. Ochratoxin also produced non-specific degenerative changes. These observations confirm previous findings with aflatoxin which are consistent with its effects on RNA metabolism. The effects of ochratoxin are similar to those observed in cell cultures exposed to ribonuclease.

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