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Fine Structural Changes and Autoradiographic Studies of Rat Liver Cells Induced by Aflatoxin B_1 and G_1

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ABSTRACT

Ultrastructural changes were induced in rat liver by the single administration of aflatoxin B_1 and G_1.

These were assessed at intervals of 1, 6, 18, 24 and 48 hours and 1, 4 and 8 weeks.

It would appear that administration of aflatoxin B_1 induced a marked nucleolar alteration seen from 6 hours after the injection. No significant alterations were noted in animals treated with a small amount of aflatoxin G_1, but injection of a larger amount of aflatoxin G_1 induced nucleolar alterations similar to aflatoxin B_1 treatment. The nucleolar change was characterized by segregation of the granular and fibrillar elements.

All three groups showed cytoplasmic changes, such as dilation of rough endoplasmic reticulum with detachment of ribosomes, hyperplasia of smooth endoplasmic reticulum, increased numbers of lipid droplets and microbodies.

Autoradiographic studies have shown the absorbtion of both aflatoxin B_1 and G_1 in parenchymal cells, but aflatoxin B_1 was absorbed more intensely than G_1, especially in the nucleus. Autoradiographic findings in electron microscopy showed a marked difference in the size grains between aflatoxin B_1 and G_1, aflatoxin G_1 being larger than aflatoxin B_1.

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INTRODUCTION

The aflatoxins are metabolites of certain strains of aspergillus flavus that grow on ground nuts and on some other foodstuffs (Sakai and Uraguchi, 1955 ; Borker, 1966 ; Hartley et al., 1963 ; Barnes, 1967 ; Smith and Mckernan, 1962), and have proved to be toxic to several animals (Newberne et al., 1964). Allcroft and Carnaghan (1962), and Asao et al. (1963) have purified the crud aflatoxins B_1, B_2, G_1 and G_2 and found that their purified compounds are closely related to each other in terms of their structures and toxic effects on the cell metabolism of several animals.

It has been demonstrated that administration of aflatoxins to a variety of animals results in an inhibition of protein synthesis due to the effect on the RNA dependent polymerase, which takes place rapidly in a matter of a few hours (Clifford et al., 1967 ; Rogers and Newberne, 1967). Although B_1 and G_1 have structural similarity with each other, namely the only difference between them is that the dihydrofuran ring of the former is replaced by a

lactone ring in G_1, it was reported that the LD_{50} of G_1 was three times higher than that of B_1 to 1 day old ducklings (Asao et al., 1963). However, G_1 is also carcinogenic when administered to the rats. (Wogan, 1968 ; Butler et al., 1969). This is also true as regards to a necrotizing does of aflatoxin in the case of a human liver cell culture system (Zuckerman et al., 1967).

The purpose of the present investigations to determine the toxicity of aflatoxin G_1 versus B_1 by comparing the ultrastructural changes in the rat liver and also by comparing the degree of cellular incorporation of B_1 and aflatoxin G_1 in liver cells as determind by autoradiographic technics.

MATERIALS and METHODS

Male rats weighing about 200—220 gm were used for the experiment. Aflatoxin B_1 and G_1 were dissolved in Dimethylformamide (DMF) and tritium labelling was achieved by the method of Dr. W. Lizinsky(1966).

Animals were sacrificed at time intervals of 1 hour, 6 hours, 18 hours, 24 hours, 7days, 4 weeks and 8 weeks after a single administration of the aflatoxin by subcutaneous injection.

For Light and Electron Microscopy

The tissues were fixed in neutral buffered formalin and sections were prepared by the standard paraffin embedding procedure and stained routinely with Hematoxylin and eosin, Periodic acid Schiff(PAS), Methyl green pyronin and Feulgen reaction were also applied.

For electron microscopy, the animals were killed by blow on the head bled immediatly by severing the jugular vein.

Specimens of the median lobe and fixed for 2 hours at 4C′ in one percent osmium tetraoxide in veronal buffer with pH of 7.4 (Palade 1952). All tissues were dehydrated in graded alcohol and embedded in Epon 812 according to standard procedures (Luft, 1961). Thin sections were cut with a LKB Ultratome then stained with uranyl acetate (Watson, 1958) and lead citrate (Millonig, 1961), and examined with the Hitachi HU 11—E electron microscope.

Autoradiographic Studies by Light Microscopy

The tissue was embedded in paraffin by standard procedures, and cut in 2-4μ thickness. The sections were deparaffinized by immersing in xylene, and rinsed with distilled water. The slides were then coated with a nuclear emuls-

Experimental schedule

Group	No. of animals	Compounds given	Dose
Group Ⅰ	21	Dimethylformamide	0.15ml DMF containing H^3 650μc.
Group Ⅱ	21	Aflatoxin B_1	0.87mg AFT-B_1 containing H^3 650μc and dissolved in 0.15ml DMF.
Group Ⅲ	21	Aflatoxin G_1	0.87mg AFT-G_1 containing H^3 650μc and dissolved in 0.15 ml DMF.
Group Ⅳ	21	Aflatoxin G_1	2.61mg AFT-G_1 containing H^3 650μc and dissolved in 0.15ml DMF.

ion, Kodak NTB-2, and stored in a refrigerator at 4'C for 30 days. The sections were developed in Kodak Dektol which was diluted 1 : 2 with water at 20'C, fixed in Kodac acid fixer, and finally washed in running water. After drying, the slides were stained by hematoxylin and eosin (Lebloned, 1965) and examined under conventional light microscope.

Autoradiographic Studies by Electron Microscopy

Preparations were made by the method of Caro and Van Tubergen (1962). This involved direct application of a loop of L-4 nuclear sensitive emulsion to mounted sections. The exposure period was 100 days. They were developed by physical developer, followed by lead citrate staining. Routine observation was with electron microscope.

RESULT

Light Microscopic Findings:

In group 2 and 4, at 6 hours there was slight swelling of periportal parenchymal cells with increased acidophilia. By 18 to 24 hours there was slight necrosis of the periportal area with a neutrophil and mononuclear cell infiltrate. (Fig. 1, 2, 3). By 1 week, there was slight bile duct proliferation.

At these intervals, after stainings with Feulgen, Methyl green-pyronine reactions were much weaker than in the normal state. The results of these studies are shown in Table 1.

Electron Microscopic Findings:

Findings at 1 hour after the injection

In groups 2 and 4, the liver cells of periportal area were studied. The rough endoplasmic reticulum (RER) stacks showed dilation and irregular cisternae. Ribosomes were decrased and scattered through the cytoplasm either singly or as clusters. These changes were more evident in group 2 than in other groups (Fig.5).

Findings at 6 and 18 hours after the injection

Nucleolar segregation was apparent in many of the nuclei in group 2 and 4. The nucleolus was compact with reduction of the granular components and fibrillar zones, but interchromatin granules were increased in number (Fig. 6, 7, 9).

Group 3 did not show any notable changes in the nucleolus at this time. The mitochondria showed swelling and exhibited irregular profiles and infolding of both inner and outer membranes, accompanied by increased dense granules and in some instances, membranous material was observed. The golgi membrene and intracisternal spaces showed dilation and increased dense particles (Fig. 10).

In group 4, there was more prominent irregular vesiculation of smooth endoplasmic reticulum(Fig. 8, 9).

The findings at 24 hours after injection

There were still a few segregations in the nucleolus in group 2 and 4. The rough endoplasmic reticulum stacks showed slight dilatation and detached ribosomes, and alteration of mitochondria could be observed in all groups (Fig. 7, 9, 10).

The findings at 4 and 8 weeks after injection

Recovery to normal conditions was almost complete except for slight changes in the cytoplasm, such as the irregular size of mitochondria and increased number of microbodies in group 4.

The ultrastructural changes in each group are summarized on Table 2.

Autoradiographic Findings:

Light microscopic findings

Group 2 (aflatoxin B_1) showed labelling in

Table 1. Light microscopic findings in the rat liver cells injection after aflatoxin B_1 and G_1

Group \ L.M. findings		Swelling	Vesiculation	Necrosis	Pyronin stain positive	Feulgen reaction positive	PAS stain positive	Bile duct proliferation
(Control) I	1 hour	−	−	−	###	###	###	−
	6 〃	−	−	−	###	###	###	−
	18 〃	−	−	−	###	###	###	−
	24 〃	−	−	−	###	###	###	−
	1 week	−	−	−	###	###	###	−
	4 〃	−	−	−	###	###	###	−
	8 〃	−	−	−	###	###	###	−
Group II (Aflatoxin B_1) 0.87mg	1 hour	−	−	−	##	###	##	−
	6 〃	+	+	−	+	##	##	−
	18 〃	##	+	+	+	##	##	−
	24 〃	+	+	+	##	###	##	−
	1 week	+	+	+	##	###	##	+
	4 〃	−	+	−	##	###	##	+
	8 〃	−	−	−	###	###	###	+
Group III Aflatoxin G_1 0.87mg	1 hour	−	−	−	###	###	###	−
	6 〃	−	−	−	##	###	##	−
	18 〃	+	+	−	##	##	##	−
	24 〃	+	+	+	##	###	###	−
	1 week	+	+	−	###	###	###	−
	4 〃	+	−	−	###	###	###	+
	8 〃	−	−	−	###	###	###	−
Group IV Aflatoxin G_1 2.6mg	1 hour	−	−	−	##	###	##	−
	6 〃	+	−	##	+	##	##	−
	18 〃	+	+	##	+	##	##	−
	24 〃	+	##	+	##	###	###	−
	1 week	+	###	+	##	###	###	+
	4 〃	−	##	+	###	###	###	+
	8 〃	−	##	−	###	###	###	−

+ : Slight ## : Moderate ### : Severe

parenchymal cells first in the cytoplasm and later heavily in the nucleus (Fig. 12).

Group 3 (aflatoxin G_1 small dose) showed diffuse labelling in parenchymal cells and did not show localized labelling in the nucleus, but there was heavy labelling in mesenchymal cells (sinusoid, kupffer cells and endothelial cell of portal area) (Fig. 13).

Group 4 (aflatoxin G_1 high dose) showed labelling of similar degree as in Group 2 but it showed localized labelling in mesenchymal cells (Fig. 14).

Electron microscopic findings

Labelling in the nucleus was noted at 6 hours in group 2 and group 4. There is a difference of the size of grain between group 2, 3 and 4 (aflatoxin B_1 and G_1) (Fig. 15, 16).

DISCUSSION

Since curd aflatoxin was purified into several

Table 2. Ultrastructure findings in the liver cells of the rat injection after aflatoxin B_1 and G_1

Group \ E.M. finding		Nucleus		Cytoplasm							
		Membrane irregularity	Nucleolar "cap"	RER dilation	Ribosome detached	SER hyperplasia	Mitochondria swelling	Mitochondria irregularity	Lysosome increase	Microbody increase	Lipid increase
Group I Control	1 hour	—	—	—	—	—	—	—	—	—	—
	6 〃	—	—	—	—	—	—	—	—	—	—
	18 〃	—	—	—	—	—	—	—	—	—	—
	24 〃	—	—	—	—	—	—	—	—	—	—
	1 week	—	—	—	—	—	—	—	—	—	—
	4 〃	—	—	—	—	—	—	—	—	—	—
	8 〃	—	—	—	—	—	—	—	—	—	—
Group II Aflatoxin B_1 0.87mg	1 hour	+	—	+	++	+	+	—	+	—	—
	6 〃	++	+++	+++	+++	++	++	+	+	+	—
	18 〃	++	++	+++	+++	++	+	+	+	+	++
	24 〃	+	++	++	++	+	+	+	+	+	++
	1 week	+	+	+	—	+	—	—	—	—	+
	4 〃	—	—	+	—	—	—	—	—	—	+
	8 〃	—	—	—	—	—	—	—	—	—	—
Group III Aflatoxin G_1 0.87mg	1 hour	—	—	+	+	+	+	—	—	—	—
	6 〃	—	—	+	+	+	++	++	—	—	—
	18 〃	—	—	+	+	+	++	++	+	—	—
	24 〃	—	—	+	+	—	+	+	+	+	+
	1 week	—	—	—	—	—	—	—	+	—	+
	4 〃	—	—	—	—	—	—	—	—	—	+
	8 〃	—	—	—	—	—	—	—	—	—	—
Group IV Aflatoxin G_1 2.61mg	1 hour	—	—	+	+	++	++	+	—	—	—
	6 〃	—	++	+	++	+++	++	+++	—	—	—
	18 〃	—	++	++	++	++	++	++	+	+	+
	24 〃	—	+	+	+	+	+	++	+	+	+
	1 week	—	—	+	—	+	+	+	+	+	+
	4 〃	—	—	—	—	—	+	+	—	+	—
	3 〃	—	—	—	—	—	—	—	—	—	—

+ : Slight. ++ : Moderate. +++ : Severe.

RER (rough endoplasmic reticulum).

SER (smooth endoplasmic reticulum).

Table 3. Percentage of nucleus in the liver cells of the rats at different dosages of H^3-aflatoxin B_1 and G_1

Group	1 hour	6 hours	18 hours	24 hours	1 week	4 weeks	8 weeks
2	32.5%	38.5%	21.1%	13.6%	8.9%	6.7%	3.4%
3	14.1%	16.3%	10.1%	9.9%	6.0%	2.6%	2.0%
4	26.1%	31.5%	18.1%	16.1%	8.7%	6.9%	3.8%

Percentage of nucleus is expressed as number of labelled per 1,000 cells.

compounds, the one which was most extensively tested in terms of toxicity to the animal is aflatoxin B_1.

Although B_1 and G_1 have structural similarity, It was reported by Asao et al,(1963) that LD 50 of aflatoxin B_1 was three times higher than that of aflatoxin G_1 to 1 day old duckling.

It is also true as regards to the necrotizing dose of aflatoxin B_1 in the case of a human liver cell culture system reported by zuckerman (1967). Clifford and Rees (1967) and wogan (1966) compared the ability of interactions of B_1 and G_1 with DNA, and found that the action is similar but qualitatively may be different, because of a spectral shift indicating that the ability of interactions of aflatoxins B_1 and G_1 with DNA molecules is different in vitro.

These results coincide with the difference in toxicity of aflatoxins B_1 and G_1 in vitro experiments.

A consideration of the structure of aflatoxins B_1 and G_1, with their five rings and carbonyl groups capable of hydrogen binding, suggested that this might be competitive to DNA hydrogen binding itself. This would be expected if such as interaction of aflatoxin B_1 and G_1 occured with DNA in vitro. B_1 is more competitive than G_1 as indicated by the evidence of spectral shift.

Our experiment shows that group 2 (aflatoxin B_1) revealed a marked nucleolar alteration after 6 hours up to 1 week.

Also group 4 (aflatoxin G_1 high dose)showed nucleolar segregation from 6 hours until 24 hours.

Ultrastructural lesions in liver cell nucleoli of rats treated with aflatoxin B_1 were described by Bernhard et al. (1965), who injected the compound into rats after partial hepatectomy. These lesions consisted of segregation of the granular and fibrillar components with the formation of so-called nucleolar "caps."

Lafarge et al. (1966) described similar effects of the toxin in partially hepatectomized rats, and studied simultaneously the development of the nucleolar lesion and nuclear RNA synthesis.

Nucleolar ultrastructural changes were also observed by Svoboda et al. (1966), who performed electron microscopy on livers of rats and monkeys treated with aflatoxin B_1.

The various agents are known to cause nucleolar segregation. Actnomycin D produced this nucleolar change in the hepatocytes, as observed by Smuckler and Benditt (1955). Svoboda and Soga (1966) and Harris et al. (1968) reported anthromycin and pyrrolizidine alkaloids to affect nucleoli of hepatocytes.

The mechanism by which aflatoxin acts is unknown. However Freidman and Wogan(1967) demonstrated that large doses of the aflatoxin produce an inhibition of RNA polymerase activity.

Clifford et al. (1967) also reported marked RNA polymerase inhibition in livers of rats with twice the LD 50 dose of aflatoxin B_1. Clifford et al. (1967), using similar preparations, found that aflatoxin G_1 also inhibited RNA synthesis, but to a lesser extent than aflatoxin B_1. In conclusion, nucleolar segregation is the ultratsructural reflection of nucleolar dysfunction. That is, alteration in the synthesis of ribosomal precursors by various agents which bind DNA and RNA synthesis and protein synthesis may also be partly responsible for the ultrastructural changes observed.

Through this experiment, autoradiographic studies have shown the incorporation of both aflatoxin B_1 and G_1 on parenchymal cells, but aflatoxin B_1 was incorporated more intensely than G_1, especially in the nucleus. Autoradiog-

raphic findings with electron microscopy showed a marked difference in the size of grains between aflatoxin B_1 and G_1, namely G_1 being larger than B_1.

However, it is not certain that this difference in size of grains is related to the storage time of aflatoxin in liver cells.

In summary, the data obtained by the present experiments indicate that both aflatoxin B_1 and G_1 exert a toxic effect upon rat liver cells. However, the degree of toxic effect of aflatoxin B_1 is much stronger than that of G_1 as judged by nucleolar segregation, and capacity for nuclear incorporation.

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Legends of Figures

Fig. 1. Rat liver, 6 hours after the injection DMF (Control group). Hematoxylin and eosin stain. ×430.

Fig. 2. Rat liver, 6 hours after the injection aflatoxin B_1. Necrosis of contigeus cells in evident along with cell debris. Hematoxylin and eosin stain. ×430.

Fig. 3. Rat liver, 24 hours after the injection aflatoxin G_1. Numerous vesicles seen in hepatocytes. ×430.

Fig. 4. Hepatic parenchymal cell, rat killed 6 hours after the injection DMF (control group).
The stacks of rough endoplasmic reticulum (rer) with ribosome (ri) and mitochondria (mi) showing normal arrangement. ×16,000.

Fig. 5. Part of nucleus (Nu) and cytoplasm of rat liver cells, 6 hours after the injection aflatoxin B_1.
Irregular shape of nucleus (Nu) and mitochondria (mi), increased number of lysosome (ly) and slightly dilation of rough endoplasmic reticulum stacks with detached ribosome. ×16,000.

Fig. 6. Part of Nucleus (Nu), 6 hours after the injection aflatoxin B_1.
The nucleolus fragmentation and segregation of granular and fibrillar components. ×20,000.

Fig. 7. Part of Nucleus (Nu) and Cytoplasm of rat liver cells, 24 hours after the injection of aflatoxin G_1 (group 3).
The fibrillar components (f) and the granular components (g) show complete segregation, and there is marked proliferation of smooth endoplasmic reticulum. ×30,000.

Fig. 8. Part of Cytoplasm of rat liver cells, 18 hours after the injection aflatoxin B_1.
The most consistent change is dilation of the cisterns with detached ribosome (arrow), and increased number of lipid droplets. ×30,000.

Fig. 9. Part of Nulceus(Nu) and Cytoplasm of rat liver cells 6 hours after the injection aflatoxin G_1(group 4).
The nucleolar "caps" fromed with dissociation of fibrill (f) and granular components (g). There is marked proliferation of smooth endoplasmic reticulum. × 8,900.

Fig.10. Part of Cytoplasm of rat liver cells, 18 hours after the injection aflatoxin G_1.
Showing irregular shape of mitochondria (mi) and contained membranous material and vesicular formation of smooth endoplasmic reticulum. ×18,000.

Fig.11. Radioautograph of rat liver cells at the control group. Paraffin section. H-E stain. ×1,800.

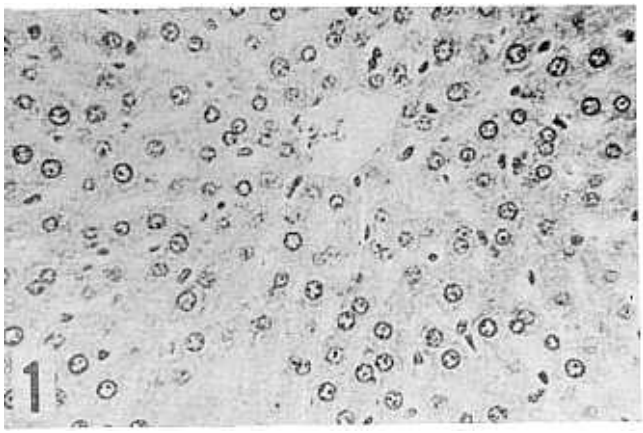
Fig.12. Radioautograph of rat liver cells at the 6 hours after given aflatoxin B_1. Paraffin section. H-E stain.
Silver grains are heavely located over nuclus. ×1,800.

Fig.13. Radioautograph of rat 6 hours given aflatoxin G_1. Paraffin section. H-E stain.
There is radioactivity in the nucleus and cytoplasm. ×1,800.

Fig.14. Radioautograph of rat liver cells at the 6 hours given aflatoxin G_1(group 4). Paraffin section. H-E stain.
Silver grains are located over nucleus ×1,200.

Fig.15. Electron microscope radioautograph of rat liver cell 6 hours after administration of aflatoxin B_1.
Numerous silver grains seen on the nucleus area. ×30,000.

Fig.16. Electron microscope radioautograph of liver rat cell 6 hours after administration of aflatoxin G_1.
Few large silver grains located nucleus and the cytoplasm ×24,000.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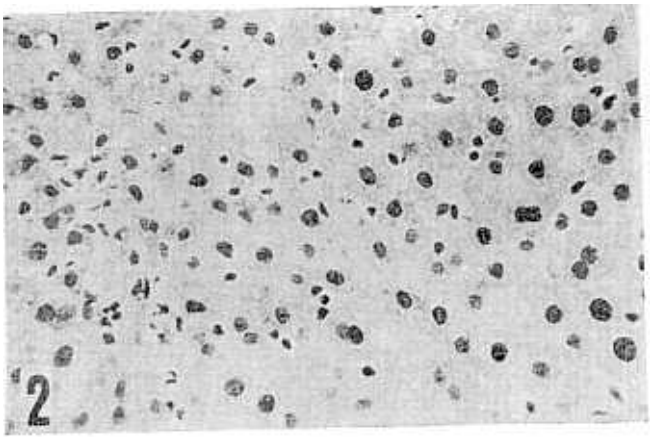
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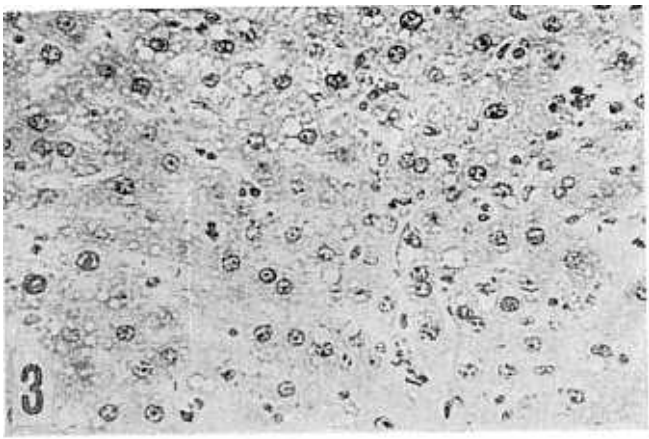
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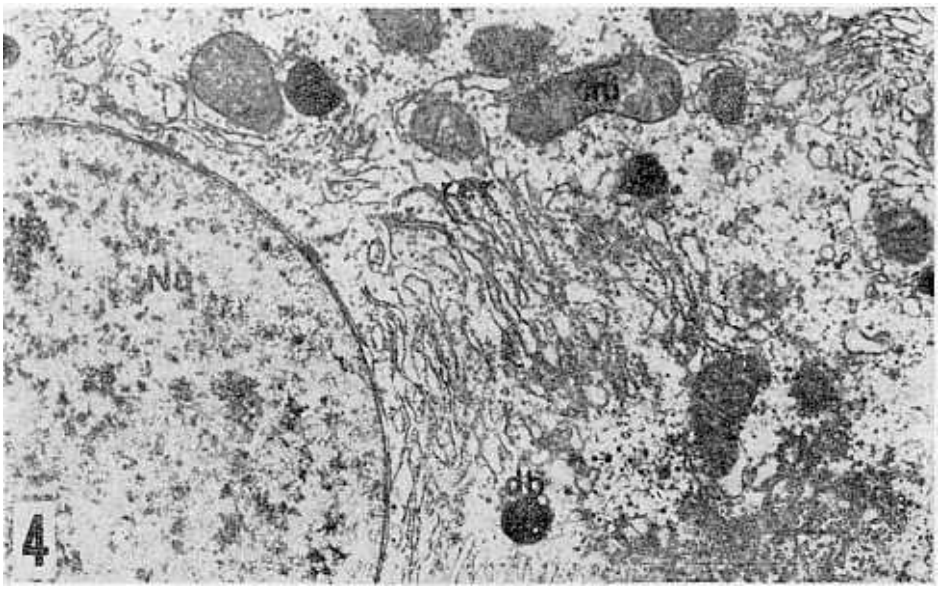
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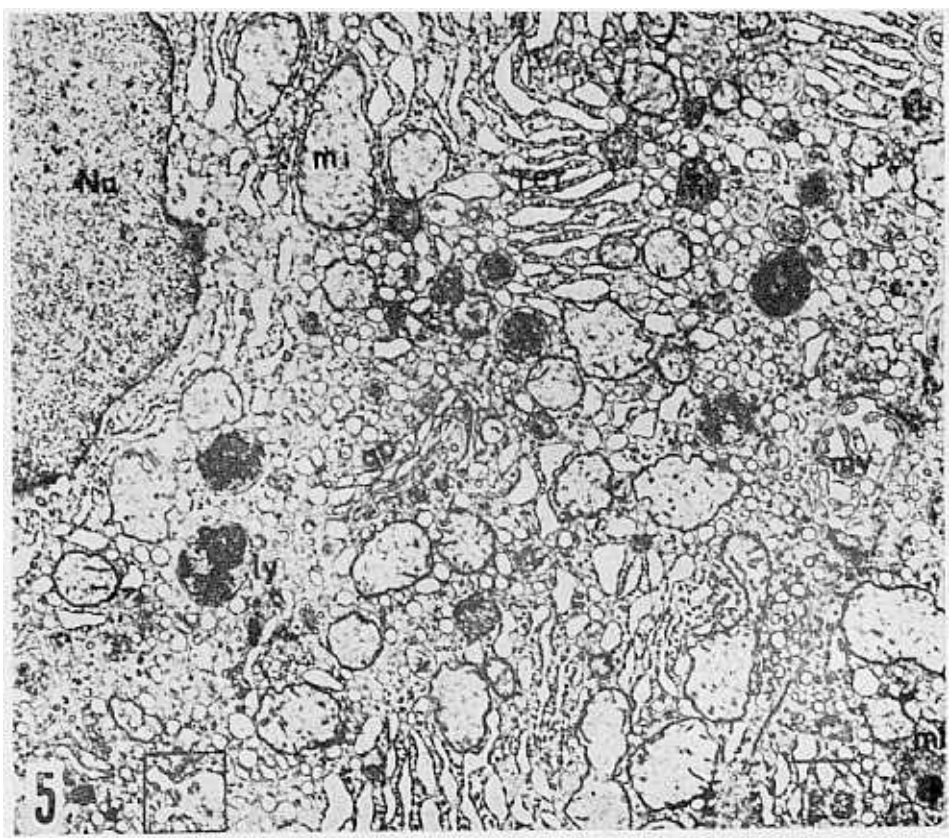
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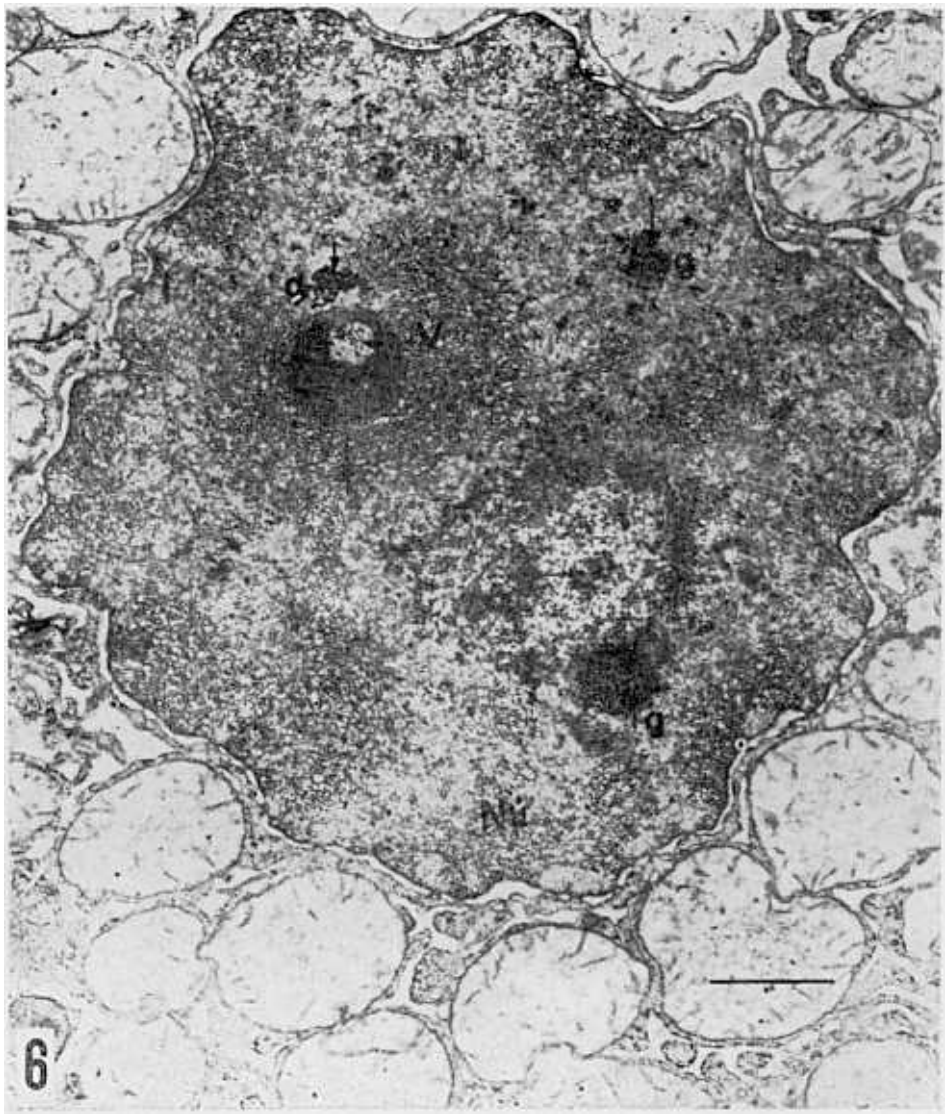
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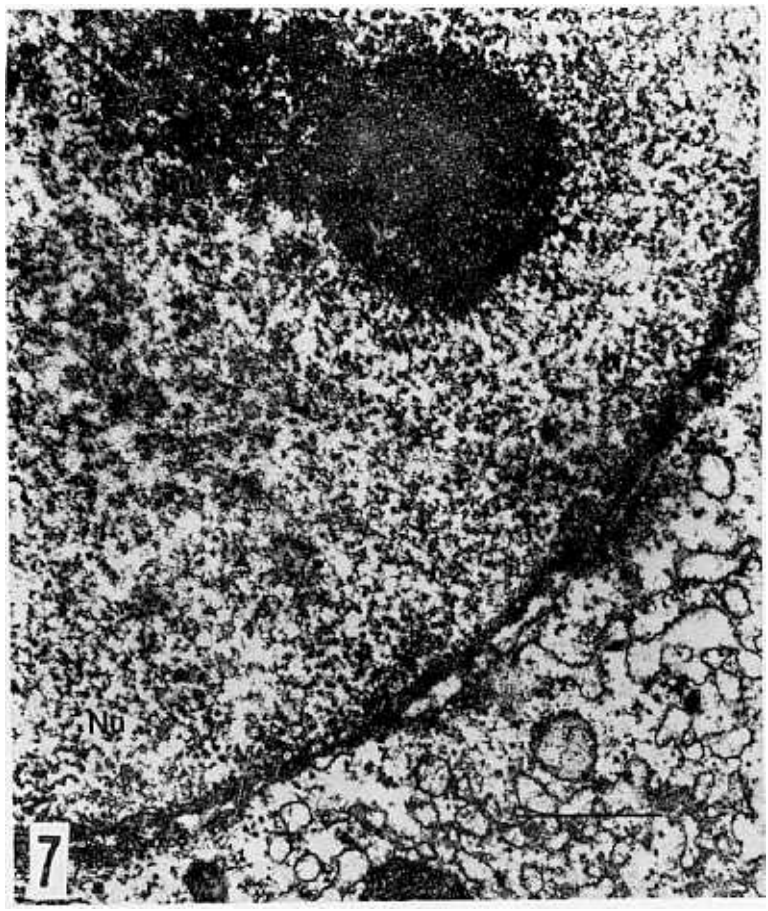
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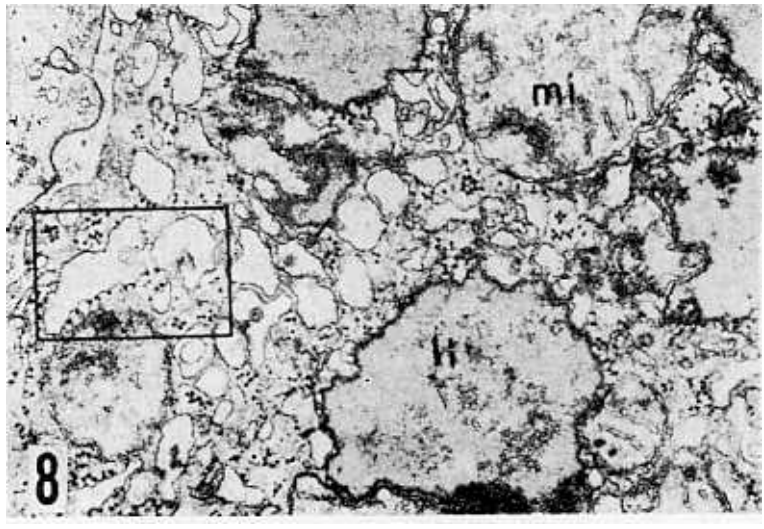
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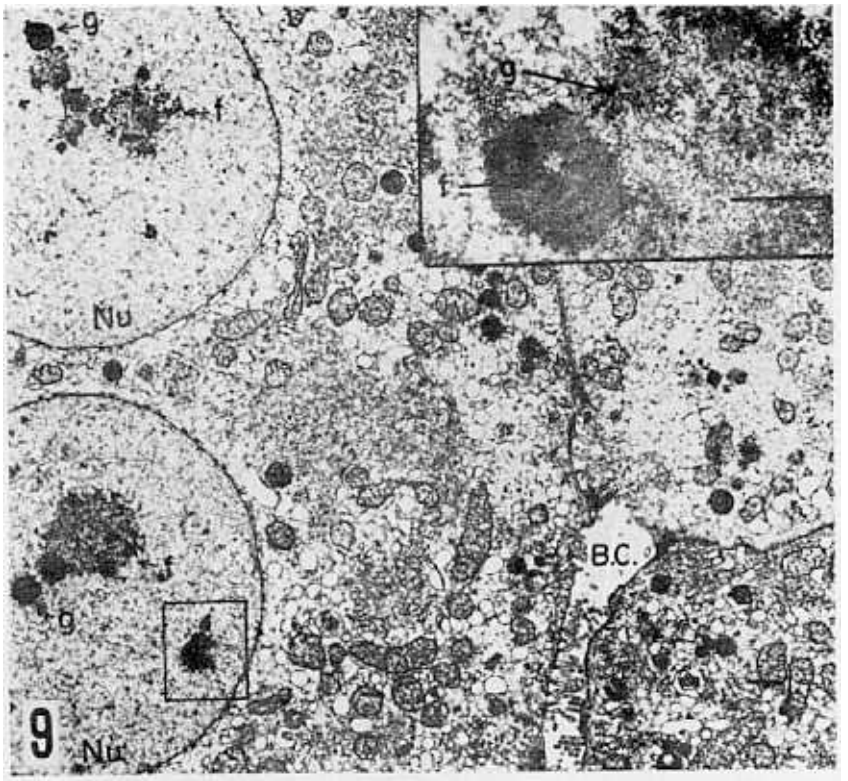
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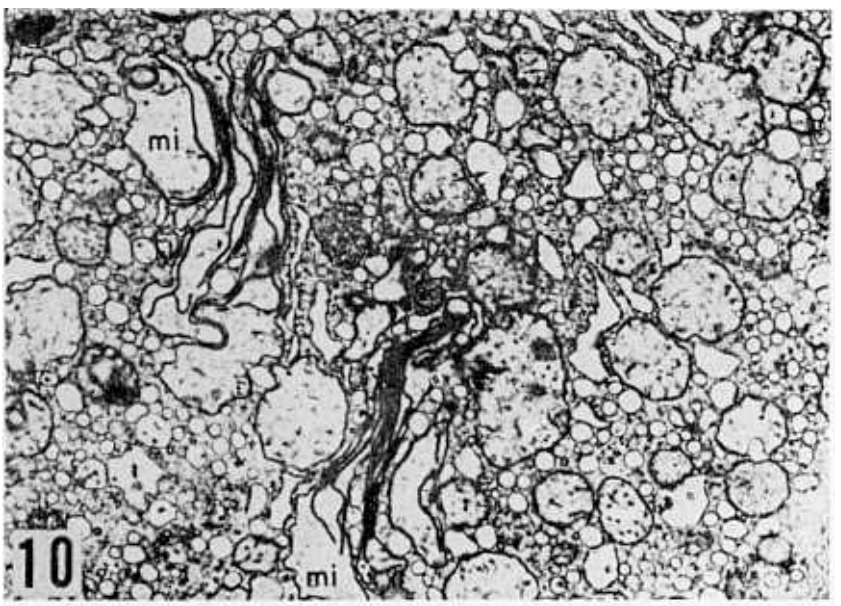
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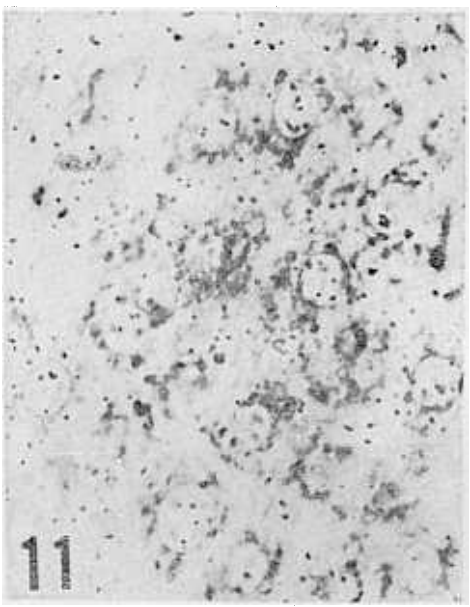
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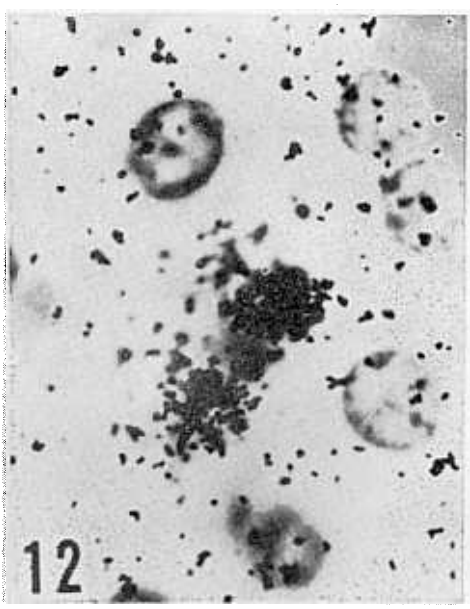
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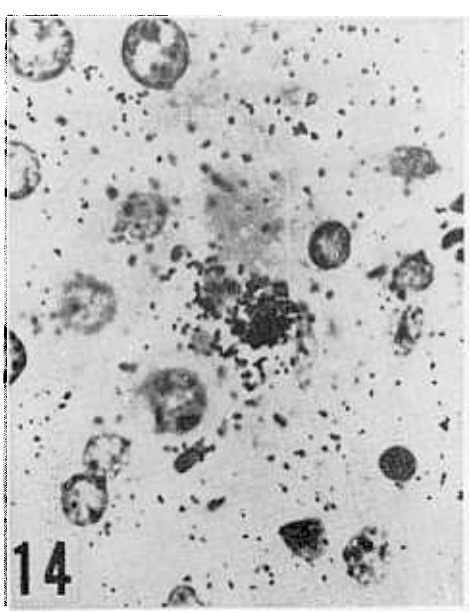
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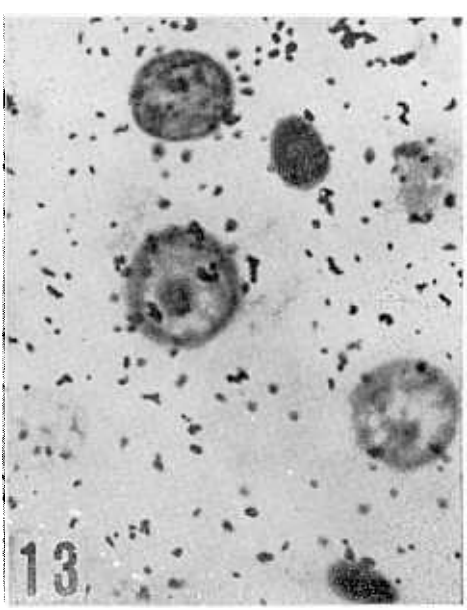
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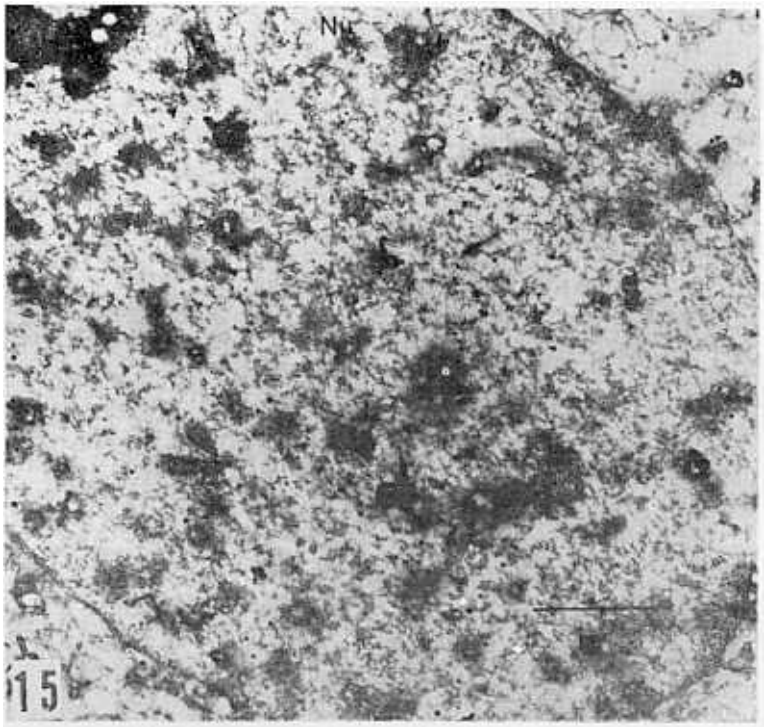
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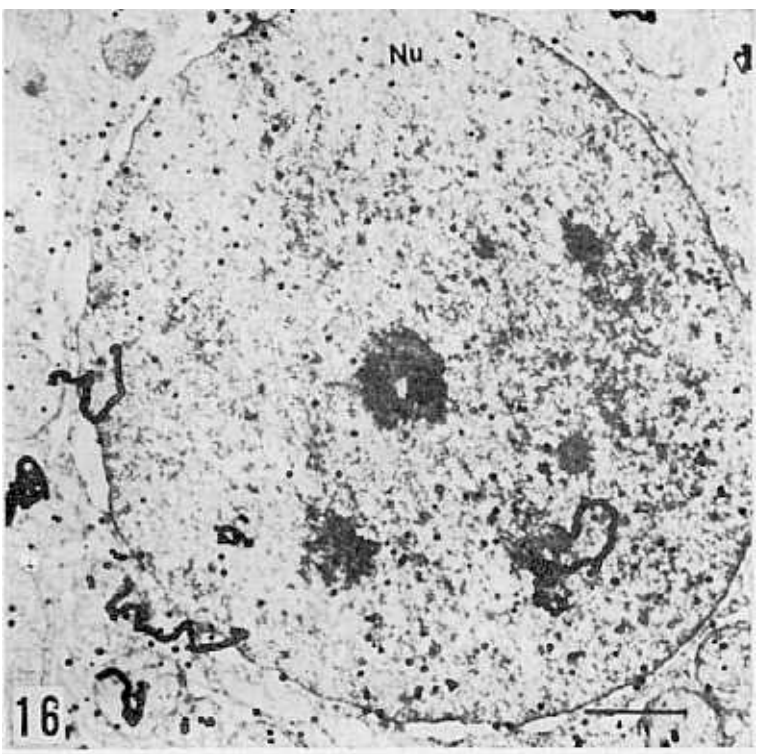
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