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Changes in Phalloidin-Sensitivity of Hepatocytes of Rats during 2-Acetylaminofluorene Carcinogenesis

Norimasa SAWADA and Hideyuki TSUKADA
Department of Pathology, Cancer Research Institute, Sapporo Medical College, Sapporo 060, Japan

SUMMARY

F-344 male rats weighing about 140-150 g were fed a diet containing 0.02% 2-acetylaminofluorene (2-AAF) for 3 or 15 weeks. Some of the rats were fed the basal diet for 5 to 6 months after the termination of the 15-week 2-AAF feeding, yielding well-differentiated hepatomas. After the carcinogen-treated hepatocytes or hepatoma cells were dispersed by a collagenase-perfusion method, the degree of phalloidin-sensitivity of the cells were assessed semi-quantitatively, using the formation of cytoplasmic blebs over the cell surface as a parameter. Furthermore, the relationship between γ-glutamyl transpeptidase (GGT)-positiveness and phalloidin-sensitivity of the cells was investigated cytochemically on an 1-hour primary culture.

Phalloidin-sensitivity decreased significantly in the order of the 3-week-treated hepatocytes, the 15-week-treated hepatocytes, and hepatoma cells, accompanying an increase in the population of GGT-positive cells. Although the sensitivity of both of the GGT-positive and GGT-negative hepatocytes was decreased as the carcinogen feeding was prolonged, GGT-positive cells were far less sensitive to phalloidin than the GGT-negative cells. Furthermore, GGT-positive hepatoma cells were also less sensitive to phalloidin than the GGT-negative tumor cells.

INTRODUCTION

Relative resistance of putative preneoplastic hepatocytes against hepatotoxins and hepatocarcinogens has been considered as one of characteristic properties of the cells which cause the cells to survive and proliferate selectively during the carcinogenesis (7, 11, 16, 27, 30). In this context, the acquisition of relative resistance of carcinogen-treated hepatocytes to various toxins or carcinogens was reported by using *in vitro* systems (5, 6, 14, 18, 23, 24, 25, 32). The *in vitro* experiments have a greater advantage over the *in vivo* experiments for the quantitative assessment of the degree of the resistance.

Phalloidin is a mushroom toxin that specifically acts on hepatocytes, inducing *in vitro* the formation of cytoplasmic blebs over the cell surface (28, 29). Since cytotoxicity of phalloidin was suggested to be related neither to its binding to ligandin (31) nor to its biotransformation by drug-metabolizing enzymes (20) within the cells, the decrease in the sensitivity of the cells was assumed to be ascribed to a decrease in cellular uptake of the toxin (32). In other words, changes in the sensitivity might indicate changes in the cell membrane functions. Thus, a decrease in the sensitivity was reported in baby rat hepatocytes (33), regenerating rat hepatocytes (33), and carcinogen-treated hepatocytes (25, 32). Furthermore, our cytochemical examinations on carcinogen-treated rat hepatocytes showed that γ-glutamyl transpeptidase (GGT)-positive hepatocytes were far less sensitive than the GGT-negative cells (25).

The purpose of the present experiments was to evaluate the changes in phalloidin-sensitivity of rat hepatocytes during 2-acetylaminofluorene (2-AAF) carcinogenesis and also of the hepatoma cells, using isolated cells and GGT-cytochemistry.

MATERIALS AND METHODS

Animals : F-344 male rats weighing about 140—150 g were fed continuously a diet (NMF, Oriental Yeast Co., Tokyo, Japan) containing 0.02% 2-AAF for 3 or 15 weeks *ad libitum*. Some of the rats were placed on the basal diet for 5 to 6 months after the termination of the 15-week 2-AAF feeding. The animals were maintained in a climatized animal quarter (23±1°C) with a fixed artificial light cycle (12 hour).

Isolation and Culture of Hepatocytes and Hepatoma Cells : Hepatocytes and hepatoma cells were isolated by a collagenase-perfusion method (3) with some modifications: 1) Perfusion through portal vein *in situ* with 37°C-warmed Ca^{2+}-free Hanks' balanced salt solution (HBSS) supplemented with 0.5 μg/m*l* insulin, 0.04 mg/m*l* streptomycin, and 40 IU/m*l* penicillin-G for 2 minutes at a flow rate of 57 m*l*/min. 2) Perfusion on a recirculation apparatus with the HBSS containing 130 units/m*l* collagenase (Sigma Chemical Co., St. Louis, Mo.) for 12 minutes. 3) Mincing with scissors. 4) Bubbling with a gas-mixture (5% CO_2-95% O_2) for 3-4 minutes. 5) Filtration through a gauze, twice. 6) Centrifugation at 50×g for 1 minute. 7) Resuspension in the HBSS containing 1.5% bovine serum albumin (fraction V) and 50 IU/m*l* mycostatin. 8) Washing with the albumin-containing HBSS three times by a centrifugation at 50×g for 1 minute. 9) Resuspension with Williams' medium E supplemented with 10% calf serum (GIBCO, Grand Island, N. Y.), insulin, streptomycin and penicillin-G, and centrifugation at 50×g for 1 minute. 10) Resuspension with the Williams' medium

E. 11) Filtration through nylon mesh, twice (Pore size: 250 and 62 μm). 12) Determination of viability of the cells by trypan blue exclusion test. 13) Preparation of cell suspension at a concentration of 2×10^5 and 6×10^5 viable cells/m*l* for assessing phalloidin-sensitivity in 35-mm plastic dishes and in test tubes, respectively. Perfusates were gassed continuously with the gas-mixture during perfusion 1) and 2). After the perfusion 1), the left branch of portal vein was ligated and the left lobe of the liver was resected and fixed in ice-cold ethanol or 10% formalin for histochemical or histological examinations. Hepatoma cells were isolated from the tumors enucleated from the collagenase-perfused livers, according to the above-mentioned procedure. Viability of the isolated hepatocytes and hepatoma cells used in this experiments was 80—95%.

Assessment of Phalloidin-sensitivity and GGT-cytochemistry : To 1 m*l* of the cell suspension containing 6×10^5 viable cells was added 1 m*l* of the medium (without addition of the serum) containing various concentrations of phalloidin (Sigma), and they were incubated at 37°C for 20 minutes with a gentle shaking. After the incubation, the mixtures were immediately cooled in ice to stop the phalloidin action (9). After adding trypan blue solution, the cells that were viable and had formed multiple cytoplasmic blebs over the cell surface were counted. The phalloidin-sensitivity was expressed as a percentage of the number of the cells with the multiple blebs to the total number of the observed viable cells. Phalloidin-induced bleb formation was observed only in viable cells but not in non-viable cells as reported by Frimmer *et al.*(10). Five-8×10^2 viable cells were counted on a hemocytometer in each examination. For GGT-cytochemical examinations, 1.5 m*l* of the cell suspension containing 3×10^5 viable cells was inoculated in 35-mm plastic dishes. After an 1-hour cultivation in a CO_2-incubator, the cells were washed three times with the culture medium and then incubated in 1.5 m*l* of the phalloidin-containing medium at 37°C for 20 minutes. After the incubation, more than 95% of the cells attached to the dishes were viable even in the presence of 10 μg/m*l* phalloidin, when examined by trypan blue exclusion test. The cells on the dishes were fixed in ice-cold ethanol for 15 minutes and stained for GGT-activity according to the method of Rutenburg *et al.*(22). Approximately 1.5×10^3 cells were counted to evaluate the percentage of GGT-positive cells under a microscope equipped with a 15× ocular and a 20× objective. Five-7×10^2 cells were also counted to assess the relationship between phalloidin-sensitivity and GGT-positiveness under a microscope using a 15× ocular and a 40× objective.

RESULTS

Histochemical examinations on the liver tissues revealed that some of hepatocytes located in peripheral zone of the liver lobules became GGT-positive in

rats fed 2-AAF for 3 weeks, though no hyperplastic lesions were found, either GGT-positive or GGT-negative (Figs. 1 and 2). The number of the GGT-positive cells was calculated as 2.28% when the isolated cells in the culture dishes were examined, in contrast to the number in normal liver (0.14%). On the other hand, in the livers of rats fed the carcinogen for 15 weeks, numerous hyperplastic nodules were induced as shown in Fig. 3. Histochemically, a portion of a nodule was found to be occupied by GGT-positive hepatocytes while another portion was GGT-negative, or, a nodule was entirely occupied by the GGT-positive cells while other nodule were almost completely negative for GGT (Fig. 4). The percentage of the GGT-positive cells was 30.38%. As shown in Fig. 5, hepatomas induced in the present experiments were well-differentiated tumors. In the tumor nodules, the GGT-positive and -negative tumor cells were present *at random* simultaneously (Fig. 6). The number of the GGT-positive tumor cells was 34.97%, when calculation was made on the cells isolated from the tumor tissues.

As described above, phalloidin-sensitivity of the cells was assessed by calculating the proportion of the cells that had formed multiple blebs over the cell surface as shown in Fig. 8 (phalloidin-treated cells) in contrast to Fig. 7 (non-treated cells). Results of the examinations on the isolated-suspended cells were summarized in Table 1. The sensitivity was observed to decrease in the order of the 3-week 2-AAF-treated hepatocytes, the 15-week 2-AAF-treated hepatocytes, and hepatoma cells.

Results of the examinations on the relationship between GGT-positiveness and phalloidin-sensitivity of the cells, with the use of GGT-cytochemistry on the 1-hour cultured cells (Figs. 9—11), were summarized in Fig. 12. The sensitivity of the

Table 1 *Changes in phalloidin-sensitivity of 2-AAF-treated hepatocytes during carcinogenesis.*

treatment period	number of experiments	percentage of phalloidin-sensitive cells / concentrations of phalloidin (μg/ml)				
		10.0	5.0	2.5	1.0	0.5
0	4	89.2[a] ±3.20	83.3 ±2.98	75.5 ±5.93	53.9 ±8.32	28.0 ±5.27
3 weeks	3	79.6 ±3.56	69.7 ±4.72	56.4 ±2.50	40.1 ±3.68	15.5 ±1.10
15 weeks	5	52.0 ±2.55	40.2 ±4.72	32.4 ±2.50	16.0 ±1.18	5.6 ±1.49
hepatoma cells	4	18.5 ±2.86	10.8 ±0.81	6.1 ±1.29	2.3 ±1.19	—

a mean±SD

cells progressively decreased for both GGT-negative and GGT-positive cells, as 2-AAF feeding was prolonged. Furthermore, the GGT-positive cells were far less sensitive than the negative cells in the preneoplastic liver as well as in hepatomas. It is worthy of note that the GGT-positive cells in 15-week 2-AAF feeding were far less sensitive to phalloidin than the GGT-positive cells found in 3-week 2-AAF feeding, and that the former cells were very similar to the GGT-positive tumor cells in the degree of the insensitivity.

DISCUSSION

Although it is expected that a few enzyme-altered foci and/or areas are induced in the liver of rats administered with 2-AAF for 3 weeks (19), we failed in the present experiments to observe the induction of these foci and/or areas in this

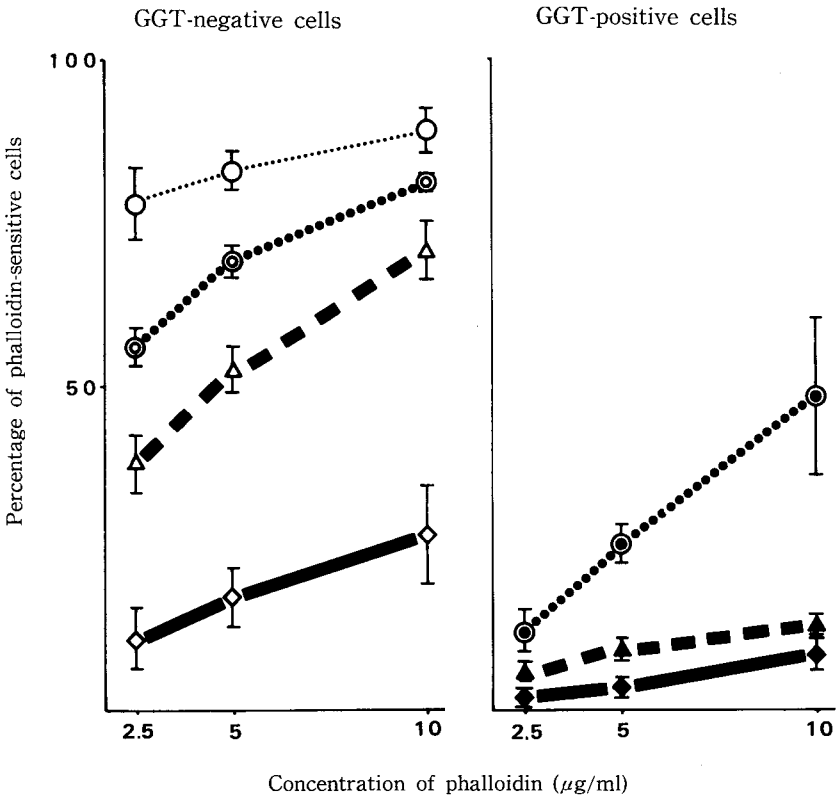

Fig. 12 Phalloidin-sensitivity of GGT-negative and GGT-positive cells during 2-AAF -hepatocarcinogenesis.

early stage of the carcinogenesis. Thus, even though a certain preneoplastic change may occur in the liver at this stage, the population of presumptive preneoplastic cells might be strikingly small to be demonstrated by the aid of the histochemical procedure. In contrast, as in the case of resistance to adriamycin (11), a decrease in phalloidin-sensitivity of the cells was found to emerge evidently already at this early stage of the carcinogen administration. It is assumed that acquisition of the relative resistance to hepatotoxins at this stage is an "adapted" or a "protective" change of hepatocytes against cytotoxicity of the carcinogen, rather than a preneoplastic change of the cells. On the other hand, at the 15 th week of the carcinogen administration, numerous hyperplastic nodules were induced. It is appropriately suggested that the resistance of the cells at this stage to phalloidin is ascribed to a preneoplastic nature of the cells.

GGT is one of the most widely used markers for putative preneoplastic hepatocytes (4, 8, 12, 13, 15, 17). The expression of GGT-activity of hepatocytes in carcinogenesis is considered as their fetal phenotypic expression and seems to suggest an alteration of the cell membranes. A decrease in phalloidin-sensitivity of hepatocytes was reported in the cells after carcinogen administration as well as in baby rat hepatocytes and regenerating hepatocytes (25, 32, 33). It is suggested that the decrease in phalloidin-sensitivity of the cells is a fetal phenotypic expression, on the one hand, and is due to an alteration of the cell membrane function to take up the toxin, on the other hand. From these aspects, the relation between GGT-positiveness and phalloidin-sensitivity of the cells during carcinogenesis was examined.

Of carcinogen-treated hepatocytes, the GGT-positive cells were found to be less sensitive to phalloidin than the GGT-negative cells as reported previously (13), and furhermore, it was found that the population of GGT-positive cells increased as the carcinogenesis advanced. It is suggested that the expression of GGT-activity of the carcinogen treated cells is at least one of the critical changes in their cell membranes related closely to the progress in preneoplastic phases. However, it might be worth emphasizing that there is a difference in phalloidin-insensitivity between GGT-positive hepatocytes isolated from rats treated with 2-AAF for 3 weeks and those obtained from the animals fed the carcinogen for 15 weeks. Another conspicuous difference between these two animal groups is that the GGT-positive cells in the former rats are found distribute in the peripheral zone of the liver lobules, while those in the latter were found in putative preneoplastic nodules. In addition, feeding rats on butylhydroxytoluene resulted in the occurrence of GGT-positive hepatocytes in peripheral zone of the liver lobules and the GGT-positive cells were found to be as insensitive to phalloidin as those seen in rats fed 2-AAF for 3 weeks, though the compound is not a hepatocarcinogen (unpublished data).

Thus, the appearance of GGT-positive, phalloidin-insensitive hepatocytes in peripheral zone of the liver lobules might not necessarily be an inevitable phenomenon in the carcinogenesis.

Previous reports (1, 2, 21) showed that hepatomas were completely resistant to phalloidin. On the other hand, in the present experiments, the tumor cells were sensitive to phalloidin to some extent, though they were far less sensitive than the preneoplastic cells. In the present experiments, only less than 40% of the tumor cells were GGT-positive, the percentage being higher than GGT-positive hapatocytes in preneoplastic nodules by some percent. Thus, insensitivity to phalloidin may be a more profitable parameter than GGT-positiveness to distinguish preneoplastic hepatocytes and hepatoma cells. However, the GGT-positive tumor cells were evidently less sensitive to phalloidin than the GGT-negative cells, suggesting the presence of a close relation between the expression of GGT and the acquisition of phalloidin-insensitivity in hepatoma cells as well.

Our working hypothesis is shown schematically in Fig. 13. Alterations in phalloidin-sensitivity, coupled with the expression of GGT-activity, suggest that there may be at least two steps in 2-AAF-hepatocarcinogenesis; non-specific changes (from A to B or C) and presumptively specific changes for the carcinogenesis (from A to I, II, or III). However, the non-specific changes seem also to be important in the carcinogenesis, since phenobarbital, a well-known liver-tumor promoter, was found to change the sensitivity from A to B (26, 31). Further experiments on phalloidin-sensitivity of hepatocytes including preneoplastic

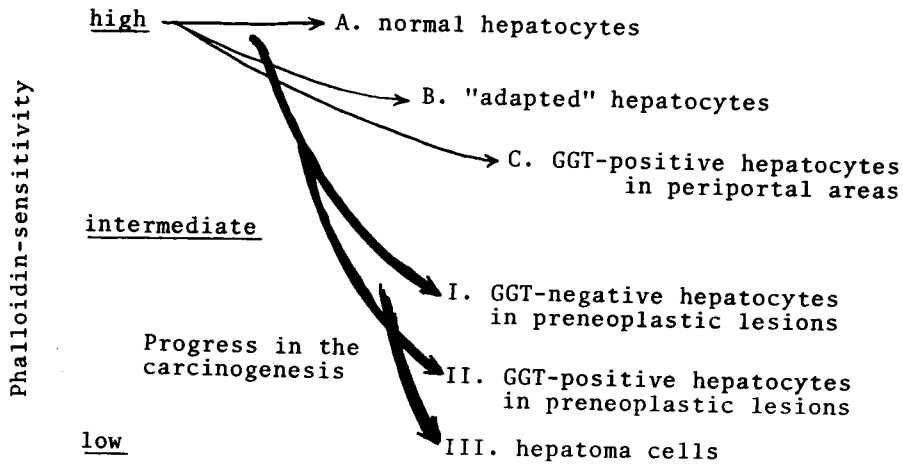

Fig. 13 Schematic diagram of 2-AAF-hepatocarcinogenesis.

hepatocytes treated with promoters are now in progress in our laboratory in order to clarfy the carcinogenesis as a multistep process.

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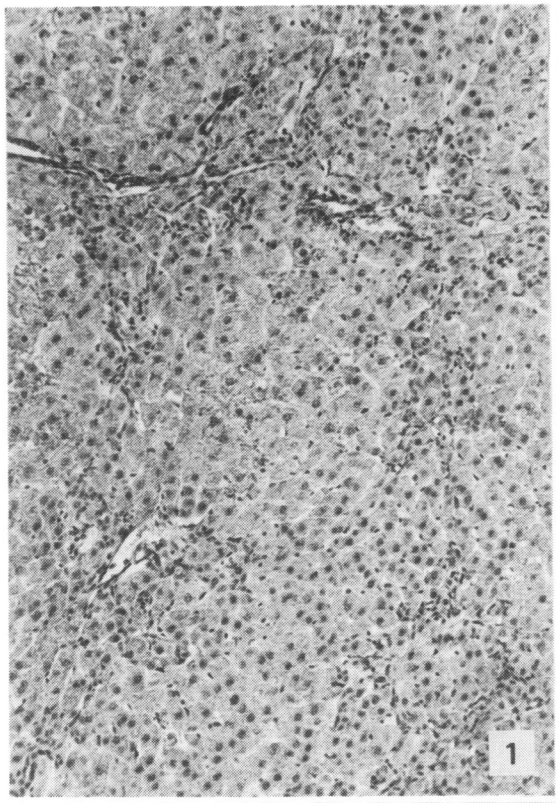
Figure legends

Fig. 1 Liver treated with 2-AAF for 3 weeks, H-E staining, ×112.
Fig. 2 Histochemistry of the section adjacent to Fig. 1, GGT-staining, ×112.
Fig. 3 Liver treated with 2-AAF for 15 weeks, H-E staining, ×45.
Fig. 4 Histochemistry of the section adjacent to Fig. 3, GGT-staining, ×45.
Fig. 5 2-AAF-induced hepatoma, H-E staining, ×448.
Fig. 6 Histochemistry of the section adjacent to Fig. 5, GGT-staining, ×448.
Fig. 7 Suspended hepatocytes isolated from normal liver without phalloidin-treatment, ×448.
Fig. 8 Suspended hepatocytes isolated from normal liver after 20-minute treatment with 10 μg/m*l* phalloidin, ×448.
Fig. 9 1-Hour cultured hepatocytes isolated from normal liver without phalloidin -treatment. ×448.
Fig. 10 1-Hour cultured hepatocytes isolated from normal liver with phalloidin-treatment (10 μg/m*l*, 20 minutes), ×448.
Fig. 11 1-Hour cultured hepatocytes isolated from 15-week 2-AAF-treated liver with phalloidin-treatment (10 μg/m*l*, 20 minutes), GGT-staining, ×690.



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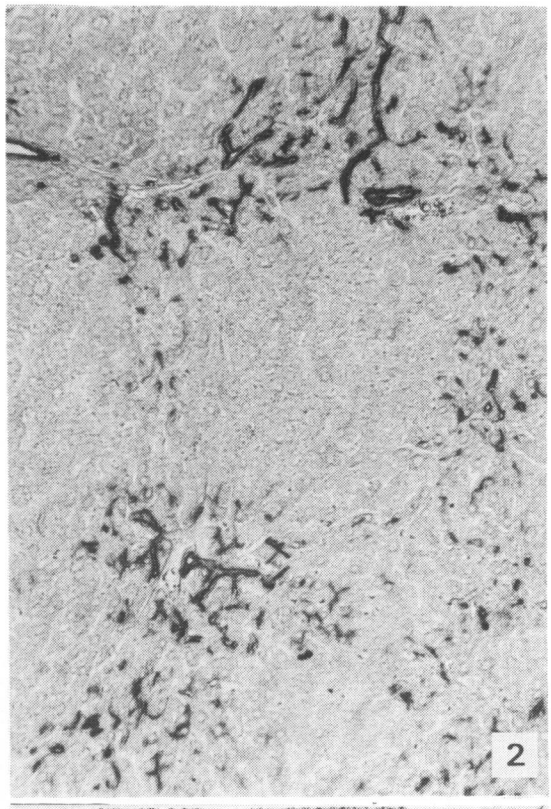

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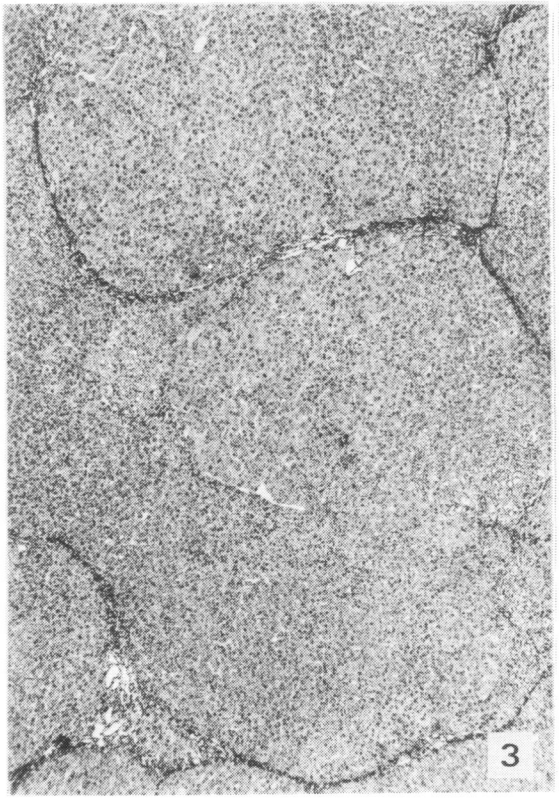

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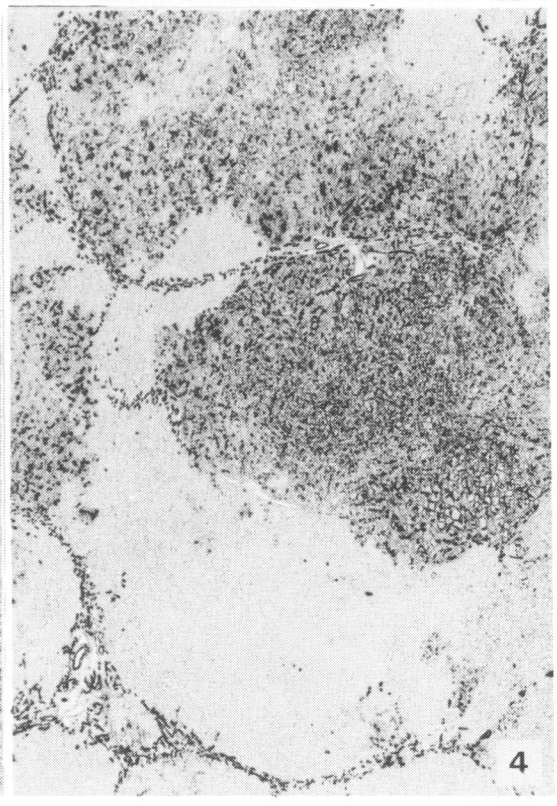

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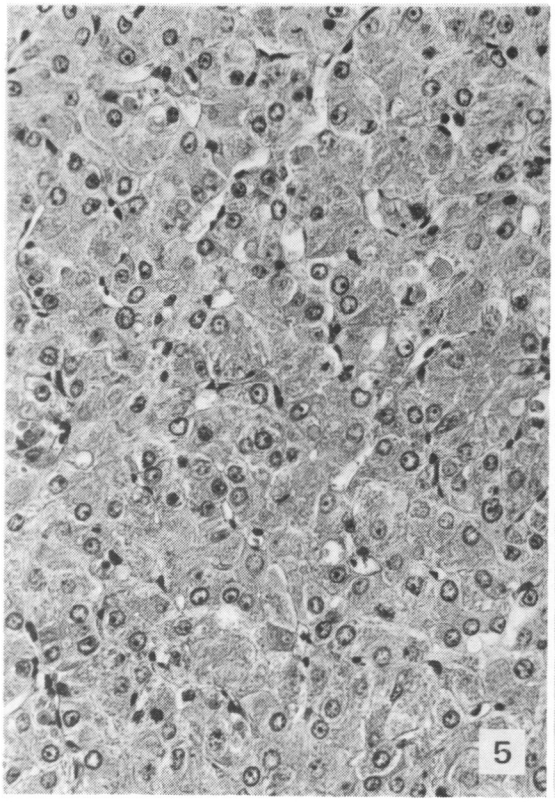

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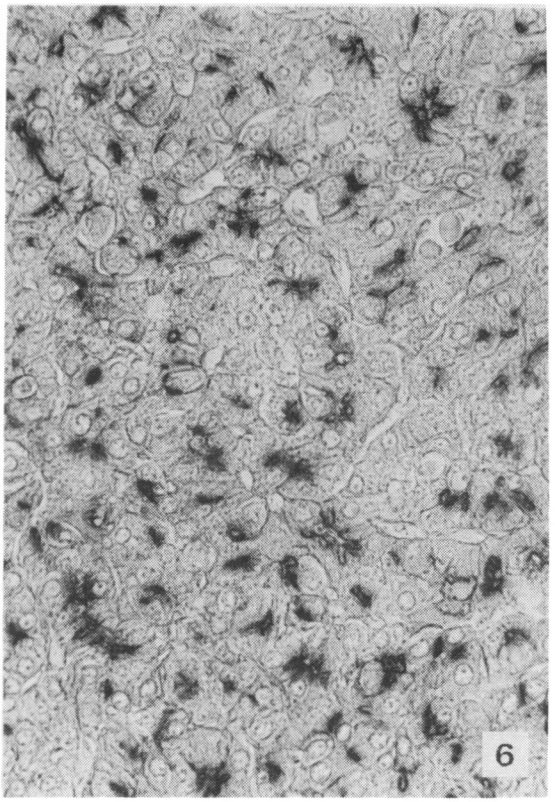

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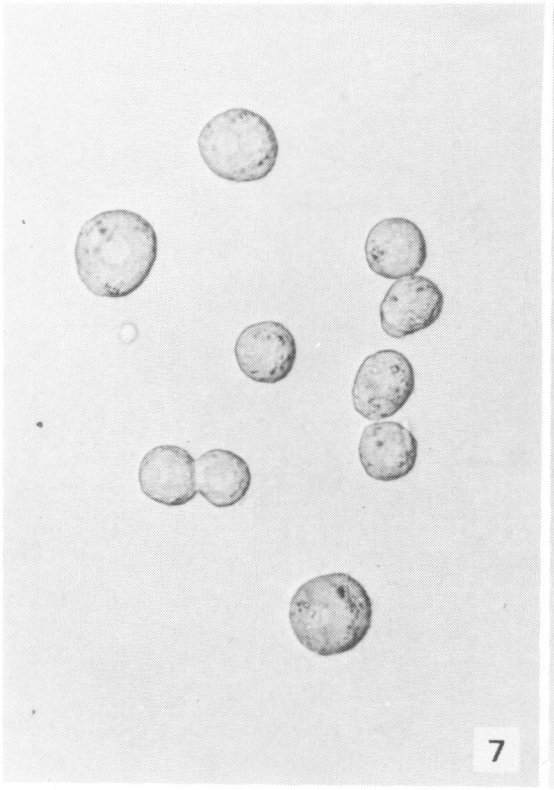

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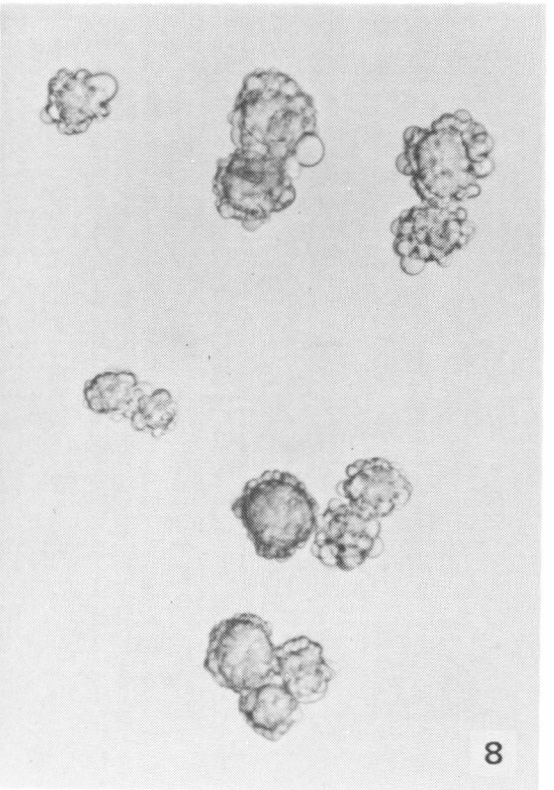

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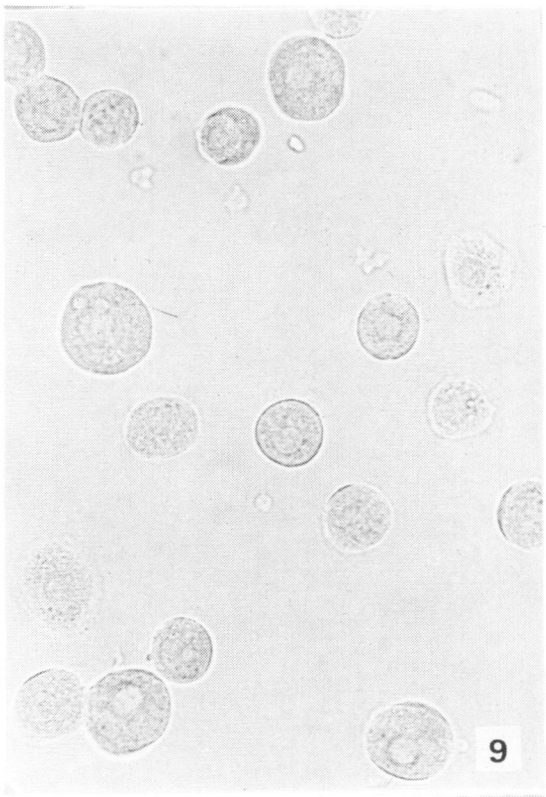

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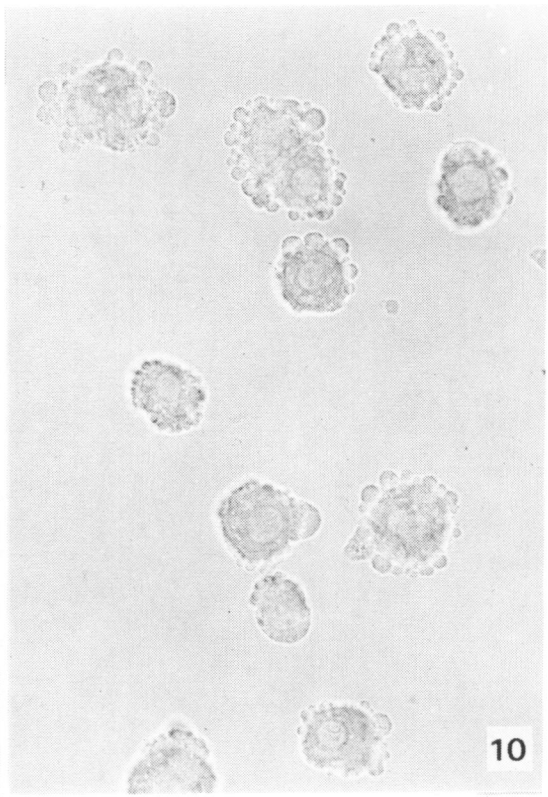

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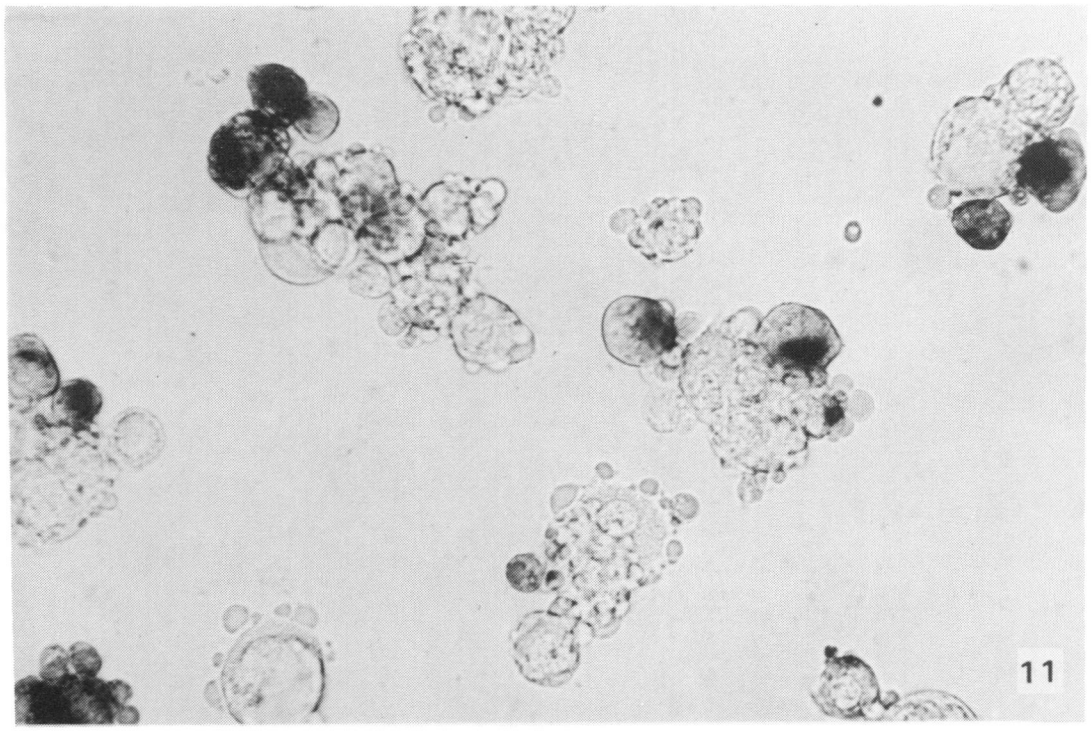

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