A Study of Cytoplasmic Lipid Granularity in Tissue Culture Cells*

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An increase in cytoplasmic granularity is considered to be one of the outstanding signs of cell injury in tissue culture. It has been used as one of the criteria of toxicity in the screening of possible antimitabolites and other substances for selective damage to neoplastic cells in vitro (7, 39). It should be recognized, however, that an increase in cytoplasmic granularity can be brought about by a variety of means not necessarily related to the agent under test. To evaluate the significance of granularity in our assay technics, it is important to determine the factors that influence its development. Some of these factors are well known (19, 21, 25, 26, 29-30, 35, 36, 41, 44).

The development of fatty granules in tissue culture cells has been shown to be hindered by a low lipid content of the medium, by serum albumin (27), or by the "anti-B" factor ("antilipofanogen") found in serum by Simms and Sanders (42).

The present study deals with the experimental production, inhibition, and reversal of cytoplasmic lipid granularity in cells grown in tissue culture and with some related cytochemical effects.

MATERIALS AND METHODS

Most of the experiments were carried out with roller-tube cultures according to the technic of Gey and Gey (20). The normal cells studied included fibroblasts from heart fragments of 2-4-day-old mice and from the abdominal skin of embryonic mice. The neoplastic cells used in this study were Crocker mouse Sarcoma 180 cells. The tissue fragments (four to six per tube) were placed in a row in glass culture tubes and then covered with a layer of equal parts of chicken plasma and 50 per cent chick embryo extract in Gey's solution. After the plasma clotted, a supernatant fluid of the following composition was added: Gey's solution, 0.4 ml.; penicillin G, 25-200 units in 0.1 ml. Gey's solution; streptomycin, 25-200 µg. in 0.1 ml. Gey's solution; horse serum, 0.2 ml.; and chick embryo extract, 0.5 ml.

For special studies, cultures were planted on coverslip inserts that were attached to the inner wall of flattened roller-tubes by means of a plasma clot. Such coverslip cultures were used in phase contrast microscopy and were made into permanent mounts for cytochemical studies.

Various gas mixtures were introduced into the culture tubes by passing the gas first through warm distilled water and then into the supernatant fluid. This procedure formed large bubbles, which filled the culture tubes and indicated roughly the amount of gas introduced. The tubes were stoppered, sealed with a paraffin-oil mixture, and incubated at 37° C. in a rotor that revolved 10 times/hour.

Test materials other than gases were incorporated into the supernatant medium by replacing an equal volume of the balanced salt solution. The wetting agent, Tween 20, was filtered through a Corning UF sintered glass filter and diluted 1:100 with Gey's solution to make a stock solution. One-tenth ml. of the stock solution was added to the supernatant fluid to give a final concentration of 1:1000 (0.1 per cent). Crystalline bovine serum albumin (Armour) in 5 per cent concentration in a balanced salt solution was used (ordinarily 0.1 ml/tube) in attempts to diminish the granularity induced by other agents. From 0.1 to 0.4 ml. of the 1.0 ml. of fluid medium was replaced with the albumin preparation in efforts to modify the granularity of Sarcoma 180 cells, which may be considerable even when the cells are rapidly proliferating.

The influence of treatment with several purines of known toxicity on granularity and on the intensity of plasma stain was studied in coverslip cultures of Sarcoma 180 and AKm mouse embryo skin. As in our standard screening test (5) the medium was made up of 2 parts Gey's balanced salt solution, 1 part chick embryo extract, and 2 parts serum. The purines used included adenine sulfate and 2,6-diaminopurine. Cultures were dosed with these agents in saline by replacing 0.1 ml. of the supernatant medium 1 day after explantation, and on the following day the cultures were examined and fixed.

In most of the studies, especially those with heart fibroblasts, the cultures were usually inspected on the 4th day and

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again at 1 week of incubation. Unless otherwise specified, the culture medium was not changed throughout the experiment.

The incidence of mitosis in culture outgrowths was determined in some experiments.

Several technics were used for the demonstration of sites of plasmalogen and lipid in the cells. The plasmal reaction of Feulgen and Voit (16), according to the preferred procedure of Hack (23), was applied to tissue cultures on coverslips. A 16-hour fixation in 0.05 M mercuric chloride was followed by staining for 14 hours in basic fuchsin-sulfurous acid, followed by two sulfurous acid washes, alcohol dehydration, and mounting in diaphane. This procedure was compared with 16-hour fixation in either 0.05 M HgCl₂ or 10 per cent neutral formalin, followed by a modified Feulgen nuclear reaction (30 minutes in 7 N HCl at 50° C., 90 minutes in fuchsin-sulfurous acid, two washes in sulfurous acid), alcohol dehydration, and diaphane mounting. The latter procedures stained nuclear structures as well as the surfaces of droplets in the cytoplasm. Sudan black B was used to stain lipid granules. A saturated solution of Sudan black B in 70 per cent alcohol according to Ackermann (1) was used as the staining solution for cultures previously treated in 3 ways. One set of coverslip cultures was fixed 30 minutes in 10 per cent formalin and then stained with Sudan black B for 10 minutes, followed by washes with 70 per cent alcohol and water, blotting, and mounting in glycerogel. Another set was treated similarly, except that a 30-minute exposure to 25 per cent acetic acid was interspersed between formalin fixation and staining. A third set was exposed for 1 day to alcohol-ether (2:1) immediately after fixation, with subsequent treatment with acetic acid before Sudan black B as in the second set. According to Ackermann (1), the acetic acid treatment makes marked lipidstainable, whereas the alcohol-ether treatment extracts all lipid.

RESULTS

SOME FACTORS INFLUENCING CYTOPLASMIC LIPID GRANULARITY IN HEART CULTURES

Aging.—An increase in cytoplasmic granularity occurred regularly in cultures more than a few days old. By the 4th day of incubation, control heart fibroblast cultures in which the medium had not been renewed displayed a moderate amount of granularity (Fig. 1). The increase in cytoplasmic granularity in aging cultures could be temporarily prevented with dialyzed serum albumin. Thus, as in Figure 2, the presence of 0.1 ml. of 5 per cent bovine serum albumin in the gassed tubes prevented the increase in cytoplasmic granularity. By the 6th day the control heart cultures showed many areas of granular cells, which were usually proximal to the fragment. The cells at the periphery of the outgrowth, however, were ordinarily clear. While cultures under nitrogen gas were all markedly granular and showed much cellular debris after a week’s incubation, gassed cultures treated with albumin showed only moderate granularity and no cellular debris. Reversal of granularity in 4-day-old cultures under nitrogen gas could be accomplished by refeeding or by adding albumin to the old medium, but not by replacing the nitrogen with oxygen. Refeeding or adding albumin on the 4th day resulted in a higher mitotic incidence on the 7th day.

Wetting agent.—Tween 20 in a concentration of 0.1 per cent caused marked granularity in heart fibroblasts by the 4th day of incubation (Fig. 5). The presence of 0.1 ml. of 5 per cent bovine serum albumin allowed less cytoplasmic granularity through the 4th day (Fig. 6). Similar results on the 7th day are shown in the low-power photomicrographs of roller tube cultures in Figures 7 and 8. Addition of albumin on the 4th day diminished the extensive cytoplasmic granularity and renewed the mitotic incidence in heart cultures initially treated with Tween 20.

THE PLASMAL REACTION IN HEART CULTURES

Aging.—In heart fibroblasts incubated for 4 days, the plasmal reaction was given to some extent by the cytoplasmic granules and more prominently by the cytoplasm around or among the droplets. In cultures that were not refed, a considerable increase in this stained material occurred by the 7th day. Less plasmalogen was evident in cultures that contained albumin added either initially or on the 4th day.

Anoxia and Tween 20.—Treatment of heart cultures with Tween 20 (Fig. 9) or maintenance under nitrogen gas caused the heaviest plasmal reaction. The addition of serum albumin either at planting or on the 4th day to such treated cultures caused a decrease in the plasmal reaction (Fig. 10), thereby giving these groups much the same appearance as the controls (Fig. 11).

The above alterations in the plasmal reaction paralleled the changes in cytoplasmic granularity as seen in living heart cultures.

Staining of cytoplasm about the granules of heart fibroblasts by means of fuchsin-sulfurous acid was not quite the same with all the several methods employed. Fixation in 0.05 M mercuric chloride, followed by exposure to decolorized basic fuchsin, gave the greatest difference in staining of granules...
between cultures treated with Tween 20 (Fig. 9) and those treated with albumin alone (Fig. 12). The difference was still present in cultures fixed with 10 per cent formalin followed by the Feulgen reaction (Figs. 13 and 14). In these cultures the nuclei were stained as well as the lipid droplets and surrounding cytoplasm. The staining of lipid droplets in formalin-fixed cultures may be attributed to the pseudoplasmal reaction, as discussed by Hack (23).

**Cytoplasmic Lipid Granularity and the Plasmal Reaction in Sarcoma 180 Cultures**

*Albumin.*—An interesting difference from the lipid granularity of heart cultures was found with Sarcoma 180 cultures. These cells showed considerable lipid granularity under all conditions. Although the presence of as much as 0.4 ml. of 5 per cent serum albumin/tube produced only a slight decrease in cytoplasmic granularity as seen with phase contrast microscopy (Figs. 15 and 16), as little as 0.1 ml. of serum albumin/tube produced a marked decrease in the amount of material stained in the plasmal reaction. Figures 17 and 18 show this effect with the strict plasmal reaction, mercuric chloride fixation followed by exposure to fuchsin-sulfurous acid, while Figures 19 and 20 show the effect in cultures fixed with mercuric chloride and stained with the Feulgen reaction to show the nuclei in addition to the cytoplasmic lipid.

*Certain Toxic Drugs.*—The rounding of Sarcoma 180 cells in cultures treated with various toxic agents made it difficult to assess changes in the amount of cytoplasmic lipid granularity. However, it appeared that cytoplasmic lipid granularity in Sarcoma 180 cells was at most slightly increased by toxic concentrations of various purines, e.g., 0.1 mM 2,6-diaminopurine (7), 1.0 mM antifolic acids, 1.0 mM phosphoramides, and 0.05 mM ethylenimino-α-triazines (8).

The intensity of the plasmal reaction in Sarcoma 180 cells was moderately increased by treatment with 0.1 mM 2,6-diaminopurine. Cultures to which adenine sulfate had been added at the protective level of 0.1 mM (6), together with the 2,6-diaminopurine, showed a diminished plasmal reaction. This concentration of adenine sulfate alone caused no appreciable change in staining.

Simultaneous administration of albumin to Sarcoma 180 cultures treated with toxic doses of 2,6-diaminopurine or adenine had the same effects on cytoplasmic lipid granularity and the plasmal reaction as it did in untreated Sarcoma 180 cells—i.e., no or little effect on granularity but a diminution in the plasmal reaction. Albumin did not release the mitotic inhibition caused by toxic purines, nor did it decrease the cytoplasmic vacuolization they caused (Figs. 21, 22).

*Diluted Medium.*—Interesting effects were noted with media of diminished fat content. In cultures grown in diluted media, with no serum or embryo extract beyond that needed to clot the plasma, the plasmal reaction was reduced in intensity. In Sarcoma 180 cells cultured in Gey's balanced salt solution, treatment with 2,6-diaminopurine produced a weaker plasmal reaction than it did in cells grown in a complete medium of 2 parts Gey's solution, 1 part 50 per cent chick embryo extract in Gey's solution, and 2 parts serum. However, the plasmal reaction in diaminopurine-treated sarcoma cultures in Gey's solution was still stronger than that in control cultures in the complete medium.

In one experiment, Sarcoma 180 cells showed almost no plasmalogen stain after growth in the complete medium for 18 hours and then exposure to ox-serum ultrafiltrate for two 18-hour periods. However, presence of 0.1 mM 2,6-diaminopurine or 4.0 mM adenine in the second change of ox-serum ultrafiltrate did cause a moderate accumulation of material staining as plasmalogen in the sarcoma cells.

**Removal of Explant**

The central necrosis of the explants in Ignatowitch's experiment (26) may have freed lipid that was the source of fat accumulated in cells grown in Ringer's solution. This possibility was tested in this laboratory by removal of explants after 1 day in culture. Cytoplasmic lipid granularity in cells of the outgrowth was increased rather than diminished by this procedure (Figs. 23, 24), thereby indicating that central necrosis of the explant is not primarily responsible for lipid granularity.

**Sudan Black B Staining in Heart and Sarcoma Cultures**

Sudan black B stains cytoplasmic granules densely throughout and, unlike the plasmal reaction, fails to stain the surrounding cytoplasm. With this exception, results of Sudan black B staining of the heart cultures were comparable to those of the plasmal reaction. It may be noted that lipid droplets in fixed cells appeared larger than those in living cells under phase contrast, as though there were some coalescence and enlargement of droplets during fixation. Treatment with albumin diminished the amount of material staining with Sudan black B (Figs. 25, 26). Exposure of the albumin-treated heart cultures to 25 per cent acetic acid after fixation in neutral formalin did not cause the cytoplasmic granules to stain any more
deeply (Fig. 27).

The cytoplasmic lipid granules of formalin-fixed sarcoma cells in 4-day-old cultures stained deeply with Sudan black B (Fig. 28). In cultures that had been treated with albumin, the cytoplasmic granules were somewhat less prominent (Fig. 29). However, if treatment with 25 per cent acetic acid for the unmasking of lipids (21) intervened between fixation and staining, there was a suggestion of more prominent staining of the cytoplasmic granules of albumin-treated cultures (Fig. 30).

Extraction with alcohol-ether (2:1) eliminated Sudan black B staining of granules in both newborn heart cells and Sarcoma 180 cells in culture.

In summary, treatment of fibroblasts with albumin diminished the content of cytoplasmic lipid droplets to similar extents as seen with phase contrast, the plasmal reaction, and Sudan black B staining. However, with Sarcoma 180 cells, the greatest diminution was seen with the plasmal reaction, an intermediate diminution with Sudan staining, and least with phase contrast. Although this may reflect differences in composition of lipid droplets between heart fibroblasts and sarcoma cells, it is perhaps also an expression of relative quantity of lipid in the two cell types.

DISCUSSION

The mechanism of fat accumulation in cells in vitro under pathological conditions (fatty degeneration) has long been debated. It was early recognized that there are two possible sources of the fat (26). Fat might appear within cells by coming from the protoplasm of the cells themselves (40) or by infiltrating from without. Endogenous fat might be released by breakdown of lipoproteins (88), perhaps under attack by proteolytic enzymes normally bound but released on injury by agents of slow death (85). The possibility of fatty infiltration from without has been criticized by Thanhauser (45), who holds that fatty degeneration may result from an unbalance of enzymes concerned with lipid metabolism in the cells.

In tissue culture, however, accumulation of fat in cells has been found to depend at least in part on a source of fat in the medium. In favor of an extracellular source of the lipid are the following: early observations by Lewis and Lewis (84) on cultures in an inorganic salt solution; a report by M. R. Lewis (88) on the uptake of stain in cultured cells fed a mixture of egg yolk and Sudan III; and an experiment by Lambert (29) showing a close agreement between fat droplet accumulation and concentration of plasma in the culture medium. Simms and Stillman (49), working with cultures of fibroblasts from adult chicken aorta rendered devoid of fat granules by repeated washing with serum ultrafiltrate, found that the “B factor” of blood plasma fractions made cells reversibly granular. Simms and Sander (42) mentioned that soap has the same effect as the “B factor,” 1 part soap/million producing detectable fat granules. Evidence that components of the “B factor,” or lipofanogens, are converted into fat granules in cultured cells was obtained by treating the cultures with lipids of different solubilities and comparing the alcohol-solubility of the resulting fat droplets in the cells (41).

Ludford (86) has reviewed the subject and appears to favor an extracellular origin of fat arising in cells growing in vitro, although some workers have stressed the intracellular origin of lipids from the breakdown of protoplasmic colloids.

Several experiments have been interpreted as supporting an intracellular source of the lipid. Ignatowitch (26) found that cultures in plasma, in defatted serum plus Ringer-Locke solution, and in Ringer’s solution alone showed similar accumulations of fat in the cells. Szantroch (44) noted that cells cultivated from embryonic chick organs in pure Ringer’s solution contained some fat. A correlation was found between the amount of fat in vivo and in vitro for particular cell types. Kleinzeller (28) held that the degenerative formation of fat from other cell constituents occurs in tissue cultures that are old or have been exposed to some toxic agent.

Of the agents we used to produce granularity, Tween 20 could be expected to contribute as a lipofanogen or lipfanogen precursor to lipid granularity. Morton, Morgan, and Parker (37), after studying the various Tweens as sources of watersoluble fatty acids for animal cells grown in synthetic media, reported that the Tweens were all definitely toxic at concentrations of 0.5 per cent and 0.05 per cent. In addition, they expected that the lipases in a natural medium, as distinguished from a synthetic medium, would hydrolyze any Tween added. Jacquez and Barry (27) have shown that a characteristic toxicity found in their experiments resulted from free fatty acids in the culture medium. It is likely that our results with Tween 20 are related to a liberation of fatty acid in the medium.

The source of lipid for the fatty granules appearing in cells under anoxia or prolonged incubation without refedding is more obscure. Several possibilities suggest themselves, including a shift in intermediary metabolism, leading to increased lipogenesis.

Chaikoff and Brown (11) point out that the course of fatty acid metabolism in a given tissue is
the resultant of the rates of a number of processes, some of which are conflicting. These include the degradation of fatty acids to 2-carbon fragments, the conversion of 2-carbon fragments to fatty acids, the conversion of 2-carbon fragments to Krebs cycle intermediates, acetoacetate, and the like. The rates at which these processes occur in a given tissue are functions of the nature and concentrations of the substrates, intermediates, enzymes, and cofactors of the enzyme systems directly concerned. They are influenced by the activity of related enzyme systems that provide substrates or remove products. Further modifications are caused by hormones that affect intermediary metabolism, including insulin (24).

Some of our results may be more readily interpreted in light of the observations of Brady and Gurin (9) on the effects of anaerobic conditions on fatty acid metabolism by a water-soluble enzyme system from pigeon liver or from mitochondrial powder. The conversion of acetate to long-chain fatty acids by this system with appropriate added cofactors proceeds at almost the same high rate anaerobically as aerobically. However, the oxidation of fatty acids by the preparations is negligible under anaerobic conditions. A resultant net increase in lipogenesis in the absence of oxygen may account in part for the augmentation of cytoplasmic lipid granularity in our tissue cultures gassed with pure nitrogen.

The reduced granularity and plasmal staining of cultures grown in dilute media suggest that serum and embryo extract of the usual complete medium contribute some of the lipid of the granules.

In healthy cells, plasmalogen (acetalphosphatide) is presumed to be disposed among the lipids and lipoproteins in the ground cytoplasm (23) or in small particulates. Brachet and Chantrenne (8) have stated that plasmalogen is a component of microsomes. Acelalphosphatides were found in about equal concentrations in the mitochondrial and microsomal fractions of rat liver homogenates by Hack (22). Our studies with dilute media indicate that some of the plasmalogen in cultured cells is derived from components of the whole medium. That plasmalogen should accompany other lipids in their coalescence into droplets in cytoplasm is to be expected. Holtfreter (25) has shown that the lipid droplets in cytolysing cells of amphibian embryos contain acetalphosphatides.

The ability of serum albumin to prevent or reverse cytoplasmic lipid granularity arising under the diverse conditions noted above has its parallels in other biological systems. Albumin protection against toxic fatty acids in culture media has been reported for rodent fibroblasts by Jacquez and Barry (27), as already noted, for tubercle bacilli (13) and for Haemophilus pertussis (40).

Just how albumin exerts its effect against fatty acids and aldehydes in tissue cultures is an open question. It may be suggested that albumin acts extracellularly through its great affinity for lipids. Although Lee and Williams (32) have shown that human serum albumin labeled with iodine-131 and injected intravenously into rats is accumulated within hepatic cells in ½ hour, Barer’s refractometric studies (2) with phase contrast microscopy of living cells indicate that albumin of the immersion medium does not enter cells unless they are damaged or dead.

It would be of interest to know whether fatty acids or other lipids which accumulate within mammalian cells under various conditions may be in themselves deleterious. It was the opinion of Carrel and Baker (10) that the growth-inhibiting factors of serum were lipoidal. The hypothesis of toxicity of lipids accumulated within cells receives support from the observation that the addition of albumin to mouse heart cultures grown under a nitrogen atmosphere or treated with Tween 20 caused not only a diminution in the considerable lipid granularity, cellular disintegration, and the plasmal reaction, but also an alleviation of the mitotic inhibition. On the other hand, albumin did not release the mitotic inhibition caused by 2,6-diaminopurine in Sarcoma 180 cells. In this case the plasmal reaction was reduced in intensity by the albumin, but the lipid granularity was not affected.

Some of our results with anoxia differ from those of Fischer, Fogh, and Jensen (17), perhaps because of differences in experimental material or method. These authors reported that oxygen tension has no influence on the rate at which fat granules accumulate in the cells of the marginal zone of tissue cultures, and that it is not possible to modify this condition by supplying them with fresh nutrient substances once the cells have become filled with fat granules. On the other hand, Zweibaum (48) indicated that oxygen does have an influence on the development of fatty granules in cultured cells.

Cytoplasmic granularity of mouse Sarcoma 180 cells in tissue culture differed from that of mouse heart fibroblasts in one primary respect: greater resistance of sarcoma cell lipid granules to the presence of albumin in the medium. With certain other mouse tumors, such as sarcoma T241 and lung tumor Ma387, it should be noted that cytoplasmic granularity is not an outstanding feature of healthy cultured cells.
Subject to the potentialities of the cell type (47), the appearance and behavior of cells in culture are greatly influenced by their environment. Cells in lipid-rich media tend to accumulate lipid. This accumulation may be reversible. It would appear that cytoplasmic lipid granularity is not always in itself a reliable criterion for the ill health of cells or for the assessment of the cytotoxicity of candidate agents in chemotherapy. An agent that is a lipofanogen would cause greater fat accumulation than one that is not, although the latter may be more toxic as measured by other criteria. One such criterion pertinent in the screening of chemotherapeutic agents against cancer cells is the inhibition of cell division. Although the removal of lipid droplets by means of albumin from anoxic or Tween 20-treated cells is accompanied by increased mitotic incidence, lipid droplets have been noted in dividing heart cells (cf. Fig. 3) as well as in many dividing cells of Sarcoma 180. Lambert and Hanes (30) remarked on the fact that fat-laden neoplastic cells in tissue culture can carry out mitosis. In her “hibernating” cultures of certain cell strains, Fjelde (18) has found that the cultures capable of new growth on feeding are those previously possessing cytoplasmic fat deposits. Doljanski (15) noted that massive accumulations of lipid droplets in the cytoplasm of cultured hepatic cells regularly developed despite very active proliferation. The lipid appeared as the cells lost glycogen, indicating to Doljanski (14) that the conditions of life in vitro had changed the direction of metabolism. In the screening of agents for selective toxicity to neoplastic cells in culture, differential inhibition of proliferation of neoplastic cells is evidently more significant than an increase in cytoplasmic lipid. Accumulation of lipid should at most be used as subsidiary evidence in the assessment of cellular damage.

A good illustration of these points is furnished by studies with 6-mercaptopurine and coenzyme A in mouse tissue cultures (4). Coenzyme A is noted as a physiological substance that prolongs the life of tissue cultures (31), 6-mercaptopurine as a nucleic acid antagonist of some value in the treatment of neoplastic disease (19). Treatment with 6-mercaptopurine, which depresses mitotic incidence in both Sarcoma 180 and mouse embryo skin cultures, especially in the former, reduces the amount of fat in the cells. Fibroblasts may retain almost none of their lipid droplets. However, simultaneous treatment with coenzyme A and 6-mercaptopurine maintains both lipogenesis and mitotic incidence at normal levels. Treatment with coenzyme A alone makes for fatty cultures with high mitotic incidence. Reliance on increased lipid granularity as evidence of toxicity would be misplaced in studies with 6-mercaptopurine and coenzyme A.

**SUMMARY**

1. Increase in cytoplasmic granularity, used as one criterion of toxicity in tissue culture screening of chemicals for selective toxicity to neoplastic cells, has been investigated. A variety of experimental conditions, including anoxia, could increase cytoplasmic lipid granularity in mouse fibroblasts in vitro.

2. In mouse fibroblasts, increase in cytoplasmic lipid granularity and in plasmalogen could be inhibited or reversed for a limited time with serum albumin or by refeeding.

3. In Sarcoma 180 cells, plasmalogen, but not the lipid granularity, was readily removable by means of serum albumin.

4. Cells with massive lipid accumulations might not be unhealthy according to other criteria, including ability to proliferate.

5. It is concluded that increase in cytoplasmic lipid granularity should be used only as a subsidiary indication of toxicity in the screening of chemical agents against neoplastic cells in culture.

**REFERENCES**


Fig. 1.—Cells of newborn mouse heart culture, 4 days after planting, with masses of highly refractile lipid droplets in cytoplasm. Phase contrast, ×727.

Fig. 2.—Cells of newborn mouse heart culture, 5 days after planting in medium containing 0.1 ml. of dialyzed 5 per cent bovine serum albumin/ml. Lipid droplets are far less abundant than in cells of Figure 1. Phase contrast, ×727.

Fig. 3.—Cells of newborn mouse heart culture, 7 days after planting in medium containing 0.1 ml. 5 per cent serum albumin/ml. Lipid droplets are abundant. Rounded cell at right is in mitosis. Phase contrast, ×727.

Fig. 4.—Cells of newborn heart culture, 7 days after planting, with no albumin added. Note large masses of lipid droplets. Phase contrast, ×727.

Fig. 5.—Cells of newborn mouse heart culture, 4th day after planting in presence of 0.1 per cent Tween 20. Note masses of lipid droplets in cytoplasm. Phase contrast, ×727.

Fig. 6.—Cells of newborn mouse heart culture, 4th day after planting in presence of 0.1 per cent Tween 20 and 0.1 ml. 5 per cent serum albumin/ml. Phase contrast, ×727.

Fig. 7.—Newborn mouse heart culture, 7th day after planting in presence of 0.1 per cent Tween 20 in roller tube. Note the marked cytoplasmic granularity and the cellular debris. ×75.

Fig. 8.—Newborn mouse heart culture, 7th day after planting in presence of 0.1 per cent Tween 20 and 0.1 ml. 5 per cent albumin/ml. Cells are relatively clear, and only slight cellular debris is evident. ×75.

Fig. 9.—Cells of newborn mouse heart culture, 4th day after planting in presence of 0.1 per cent Tween 20. Mercuric chloride fixation, fuchsin-sulfurous acid staining. Large masses of cytoplasmic lipid are stained. ×675.

Fig. 10.—Cells of newborn mouse heart culture, 4th day after planting in presence of 0.1 per cent Tween 20 and 0.1 ml. 5 per cent bovine serum albumin. Mercuric chloride fixation, fuchsin-sulfurous acid staining. There is much less stained material than in cells of Figure 9. ×675.

Fig. 11.—Cells of newborn mouse heart culture, 4th day after planting in standard medium. Mercuric chloride fixation, fuchsin-sulfurous acid staining. These controls resemble cells of Figure 10. ×675.

Fig. 12.—Cells of newborn heart culture, 4th day after planting in presence of 0.1 ml. 5 per cent serum albumin/ml. Mercuric chloride fixation, fuchsin-sulfurous acid staining. There is less stained material than in the standard medium control cells of Figure 11. ×675.

Fig. 13.—Cells of newborn heart culture, 4th day after planting in presence of 0.1 per cent Tween 20. Formalin fixation, Feulgen staining. Nuclei are stained by the Feulgen nuclear reaction; large cytoplasmic lipid masses are stained by the pseudoplasmal reaction. ×675.

Fig. 14.—Cells of newborn heart culture, 4th day of incubation in presence of 0.1 ml. 5 per cent serum albumin/ml. Formalin fixation, Feulgen staining. Nuclei are stained by the Feulgen nuclear reaction but essentially no cytoplasmic lipid is stained. Note the mitosis. ×675.

Fig. 15.—Sarcoma 180 cells in control culture, with masses of refractile lipid droplets in cytoplasm, 3d day of incubation. Phase contrast, ×727.

Fig. 16.—Sarcoma 180 cells on 3d day of incubation in presence of 0.4 ml. of 5 per cent serum albumin/ml. These cells show no reduction in quantity of cytoplasmic lipid from cells of Figure 21, in spite of the albumin. Phase contrast, ×727.

Fig. 17.—Sarcoma 180 cells in control culture, 4th day of incubation. Mercuric chloride fixation, fuchsin-sulfurous acid staining show masses of cytoplasmic lipid. ×675.

Fig. 18.—Sarcoma 180 cells on 4th day of incubation in presence of 0.4 ml. 5 per cent serum albumin/ml. Mercuric chloride fixation and exposure to fuchsin-sulfurous acid reveal no material stained in plasmal reaction. ×675.

Fig. 19.—Sarcoma 180 cells in control culture, 4th day of incubation. Mercuric chloride fixation, Feulgen staining. Nuclei stained by Feulgen nuclear reaction, cytoplasmic lipid masses stained by plasmal reaction. ×675.

Fig. 20.—Sarcoma 180 cells on 4th day of incubation in presence of 0.4 ml. 5 per cent serum albumin/ml. Mercuric chloride fixation, Feulgen staining. Nuclei are stained by Feulgen nuclear reaction but cytoplasm contains only small masses of lipid weakly stained by plasmal reaction. ×675.
FIG. 21.—Sarcoma 180 cells exposed for 1 day to 1 mM adenine sulfate, a moderately toxic dose, with consequent cytoplasmic vacuolization. Phase contrast, ×727.

FIG. 22.—Sarcoma 180 cells exposed for 1 day to 1 mM adenine sulfate plus 0.2 ml. 5 per cent serum albumin/ml. Cytoplasmic vacuolization remains the same as in cells of Figure 21. Phase contrast, ×727.

FIG. 23.—Cells from periphery of newborn heart control culture, 5th day of incubation. Each cell has only a few small groups of lipid droplets. Phase contrast, ×727.

FIG. 24.—Cells from periphery of newborn heart culture, 5th day of incubation. The central explant was removed after 1 day of incubation. Each cell has larger deposits of cytoplasmic lipid than have cells of Figure 23. Phase contrast, ×727.

FIG. 25.—Cells of newborn heart control culture, 4th day of incubation. Formalin fixation, Sudan black B staining. The stained lipid droplets of several cells are seen in this photograph. ×675.

FIG. 26.—Cells of newborn heart culture, 4th day of incubation in presence of 0.1 ml. 5 per cent serum albumin/ml. Formalin fixation, Sudan black B staining. Much less sudanophilic material than in Figure 25. ×675.

FIG. 27.—Cells of newborn heart culture, 4th day of incubation with 0.1 ml. 5 per cent serum albumin/ml. Formalin fixation, acetic acid treatment, Sudan black B staining. No increase over the almost negligible sudanophilia of Figure 26. ×675.

FIG. 28.—Sarcoma 180 cells of control culture on 4th day of incubation. Formalin fixation, Sudan black B staining. Cytoplasmic lipid masses are strongly sudanophilic. ×675.

FIG. 29.—Sarcoma 180 cells, 4th day of incubation in presence of 0.4 ml. 5 per cent serum albumin. Formalin fixation, Sudan black B staining. Cytoplasmic sudanophilia is reduced from that of cells in Figure 28 but by no means eradicated. ×675.

FIG. 30.—Sarcoma 180 cells, 4th day of incubation in presence of 0.4 ml. 5 per cent serum albumin/ml. Formalin fixation, acetic acid treatment, Sudan black B staining. Cytoplasmic sudanophilia is intermediate between that in Figure 28 and that in Figure 29. ×675.
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