Studies on a Transplantable Chicken Tumor (RPL-12 Lymphoma)

III. Cytological Changes during Virus-induced Oncolysis

ROBERT LOVE AND GEORGE R. SHARPLESS

The histological changes following inoculation of the RPL-12 lymphoma into the pectoral muscle and brain of the chicken have been described (29, 30). Superimposed infection of the host with the N.F.T. strain of St. Louis encephalitis virus results in the development of prominent cytoplasmic “inclusions” in the tumor cells during the period when the virus concentration in the tumor is highest (30). Apart from some displacement or indentation of the nucleus by the “inclusions” and occasional margination of the chromatin, there is no further morphological evidence of degeneration before the tumor cells in the pectoral muscle undergo phagocytosis. When the virus is inoculated into the host with tumor growing in the brain, phagocytosis is deficient, and an abnormally large number of metaphase mitotic figures are observed in the tumor cells containing “inclusions” (30). The present experiments were designed to analyze the formation and structure of the so-called “inclusions,” and to determine the effect of virus infection on the mitotic process by comparing the cytological and cytochemical properties of the tumor cell before and after infection with virus.

MATERIALS AND METHODS

Complete details of the biological procedures and the plan of the experiments have already been published (29, 30).

Tissues.—In addition to the routine histological examination of all organs and tissues, the following material was specially prepared for cytological and cytochemical study: (a) pectoral muscle and brain tissue containing tumor, daily from the time of inoculation of virus until tumor cells in the muscle had undergone phagocytosis, or until the birds with tumors in the brain had died; (b) uninfected tumor tissue on the same days as (a).

Preparation of the tissue.—Thin fragments were scraped with a sharp knife from the cut surface of the tissue and smeared on slides. When no fixative was used (Table 1), the smears were allowed to dry for 5 minutes before staining; when fixation was desired, the slides were immersed in the fixative while still wet. Small pieces were placed in 0.85 per cent NaCl or mixed with supravital staining solutions on slides and examined directly by ordinary and phase microscopy. Blocks of tissue were fixed as described below, and paraffin and frozen sections were prepared. Except where otherwise stated (Table 1), the final preparations were mounted in Permount, and the paraffin-imbedded material which had been fixed in formol sublimate was treated with iodine and thiosulfate before staining.

Fixation and staining procedures.—Paraffin sections of material fixed in formol sublimate were stained with Barrett’s stain (4) to observe the general cytological detail. For the demonstration of Golgi bodies and mitochondria, blocks were fixed and prepared by standard methods: (a) Cajal’s silver and the Sjovall and Kolatchew osmic acid methods for Golgi bodies (33) and (b) Altmann’s and Regaud’s methods for mitochondria (35). In addition, the more recent method of Harman (22) was applied to blocks fixed in Regaud’s fluid. Supravital staining properties were investigated by placing small fragments of tissue in freshly prepared mixtures of stock solutions of 0.006 per cent Janus green and 0.01 per cent neutral red in 0.85 per cent NaCl in the proportions of 2:1, 1:1, and 1:2. A comparison of the sudanophilic with the supravital stainable material in the cells was made by replacing the supravital stain under the cover slip with Baker’s Sudan black B (1) and differentiating with 50 per cent alcohol.

To minimize repetition and clarify the rationale, the cytochemical procedures are outlined in Table 1.

Analysis of mitosis.—Owing to the rapid phagocytosis of infected tumor cells in the pectoral muscle, only brain material was available for sustained analysis of the mitotic process in sections stained by the Feulgen method. Beginning on the day when inclusions were first observed, 1,000 tumor cells in each section were analyzed, the number of cells in the various phases of mitosis was noted, together with the proportion of binucleate cells and the incidence of gross mitotic abnormalities as defined by Glucksman (17). Multipolar division, bridge formation, and lagging of an occasional chromosome in anaphase were not regarded as abnormal for the purposes of the count, since they do not necessarily lead to death of the daughter cells.

RESULTS

UNINFECTED TUMOR CELLS

Characterization of the structures of the cell.—Supravital staining with neutral red and Janus green revealed numerous rounded green mitochondria of regular size distributed throughout
TABLE 1

CYTOCHEMICAL PROCEDURES

<table>
<thead>
<tr>
<th>Purpose to Investigate</th>
<th>Fixation</th>
<th>Preparation</th>
<th>Staining</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid</td>
<td>F. sub., f. calc. and none</td>
<td>Fros. and smear</td>
<td>Sudan black B (1)</td>
<td>All material for lipid investigation, and for comparison with any lipid stain, mounted in glycerine jelly.</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>F. calc. and none</td>
<td>Fros. and smear</td>
<td>Sudan black B after lipid extraction</td>
<td>Lipid extracted by 6 hours' immersion of slides in hot chloroform-methanol.</td>
</tr>
<tr>
<td>Neutral and acidic lipid</td>
<td>F. sub. and f. calc.</td>
<td>Fros. and smear</td>
<td>Sudan black B</td>
<td>To insure that sudanophilia was not due to chemical combination with Sudan black it was shown that the dye, but not the lipid, could be removed by acetone (90).</td>
</tr>
<tr>
<td>Fatty aldehydes and precursors</td>
<td>None</td>
<td>Smear</td>
<td>Schiff (54)</td>
<td>Compared with preparations stained with Sudan dyes to confirm the lipid nature of the material stained.</td>
</tr>
<tr>
<td>Ununsaturated fatty acids</td>
<td>F. sub., f. calc. and none</td>
<td>Smear</td>
<td>Performic acid Schiff (95)</td>
<td>Direct Schiff for preformed aldehyde; planum for acetal phosphates, and Schiff after exposure to air for 3 days for aldehyde secondary to oxidation of unsaturated fatty acids (30).</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>F. calc. and none</td>
<td>Fros. and smear</td>
<td>Schults (99)</td>
<td>Mouse adrenal stained simultaneously as a positive control.</td>
</tr>
<tr>
<td>P.A.S.-positive material</td>
<td>F. sub., f. calc. and none</td>
<td>Par. fros. and smear</td>
<td>P.A.S. (94)</td>
<td>In all cases where P.A.S. stains were used, control sections of the same material were treated identically except that oxidation with periodic acid was omitted.</td>
</tr>
<tr>
<td>1,2-Glycol groups</td>
<td>F. sub. and f. calc.</td>
<td></td>
<td>P.A.S. after acetylation in pyridine (98)</td>
<td>Control section stained after treatment with pyridine alone to determine reduction in P.A.S. stain due to solution of cell constituents in pyridine.</td>
</tr>
<tr>
<td>P.A.S.-positive lipid</td>
<td>F. sub. and f. calc.</td>
<td></td>
<td>P.A.S. after lipid extraction</td>
<td>The lipid extraction process did not affect the intensity of the P.A.S. reaction of control sections of clam. A difference between the unextracted and the extracted material must be due to P.A.S.-positive lipid.</td>
</tr>
<tr>
<td>P.A.S.-positive (unsaturated) lipid</td>
<td>F. sub. and f. calc.</td>
<td>Smear</td>
<td>P.A.S. followed by Sudan black B</td>
<td>To determine whether location of the lipid coincides with that of the P.A.S.-positive material.</td>
</tr>
<tr>
<td>Acid mucopolysaccharides</td>
<td>F. sub., F. calc.</td>
<td>Smear</td>
<td>Lipid extraction, hyaluronidase (27), P.A.S.</td>
<td>Myelin stained simultaneously as a positive control.</td>
</tr>
<tr>
<td>Glycogen</td>
<td>F. sub.</td>
<td>Par.</td>
<td>Lipid extraction diastase (27), P.A.S.</td>
<td>Activity of enzyme shown by its ability to reduce metachromasia of clam acid mucopolysaccharides.</td>
</tr>
<tr>
<td>DNA</td>
<td>F. sub.</td>
<td>Par.</td>
<td>Feulgen (97)</td>
<td>P.A.S. reaction of control section of liver glycogen reduced by malt diastase preparation used.</td>
</tr>
<tr>
<td>RNA</td>
<td>F. sub.</td>
<td>Par. and smear</td>
<td>MBE (97) and 0.01 per cent toluidine blue (95)</td>
<td>Counterstained with fast green PCP.</td>
</tr>
<tr>
<td>Acid mucopolysaccharides</td>
<td></td>
<td></td>
<td></td>
<td>Stained before and after incubation for 1 hour in buffered ribonuclease (97); staining of control sections unaffected by incubation in buffer alone.</td>
</tr>
<tr>
<td>Tyrosine, tryptophan, histidine, ? purines and pyrimidines</td>
<td></td>
<td>Par.</td>
<td>Coupled tetrazoniunm (10)</td>
<td>Association of stainable material with lipid constituents of the cell investigated by staining with toluidine blue after lipid extraction. Fixation of toluidine blue stain by the method of Bensley (5).</td>
</tr>
<tr>
<td>Histone</td>
<td>Susa</td>
<td>Par.</td>
<td>Bensoylation (in pyridine) tetrazonium (10)</td>
<td>For amino acids and ? purines and pyrimidines.</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>Acetone, 15 per cent formalin or none</td>
<td>Smear</td>
<td>White's aniline blue, orange G (45)</td>
<td>For residual staining after blocking amino acids.</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Acetone or none</td>
<td>Smear</td>
<td>Gomori (18)</td>
<td>Destruction of RNA by strong acid for 16 hours (15), and of amino acid reacting groups by benzoylation.</td>
</tr>
<tr>
<td>Succinic dehydrogenase</td>
<td>None</td>
<td>Smear</td>
<td>Blue tetrazonium (40)</td>
<td>To assess amount of stainable material removed by pyridine.</td>
</tr>
<tr>
<td>A ascorbic acid</td>
<td>None</td>
<td>Smear</td>
<td>Modified Bourne (97)</td>
<td>Since results were negative, cells from the Ehrlich ascites tumor which were known to be positive were run simultaneously as controls.</td>
</tr>
</tbody>
</table>

*Section of clam in which presence of acid mucopolysaccharides and alkaline phosphatase and absence of lipids was demonstrated (97).

the cytoplasm and a small number of slightly larger irregularly sized neutral red staining particles, mainly located in the nuclear hof. A few large refractile globules in the cytoplasm did not stain. Instillation of Sudan black stain beneath the cover glass of supravital preparations clearly demonstrated the lipid nature of the refractile globules, which stained in a few seconds, and of the mitochondria, which were visibly stained after about 10 minutes. Unfortunately, the Sudan black solution rapidly destroyed the supravital staining properties of the cell, and there was a latent period when the cell was unstained and considerable shrinkage occurred. It was not possible, therefore, to visualize the exact fate of every neutral red particle. A comparison of drawings of the supravital stained and the Sudan black-stained cell revealed that some of the neutral red vacuoles were probably identical with sudanophilic granules which became visible at about the same time as the mitochondria, but were mostly confined to the nuclear hof.

Phase contrast microscopy confirmed the existence of spherical particles of variable size in the cytoplasm but did not distinguish different types. In sections stained by Altmann's, Regaud's, and Harman's methods for mitochondria, and the Cajal silver, Sjovall, and Kolatchew osmic acid methods for Golgi bodies, the results were variable: cytoplasmic particles corresponding to the Janus green and the neutral red staining material were present in parts of sections stained by all methods except the first, in which only a few coarse granules could be seen. In the less intensely stained preparations only the smaller granules were stained, while, as the density of staining increased, the larger granules stained around the periphery and finally appeared as solid bodies. Even in the most intensely stained preparations scanty unstained vacuoles were present in the peripheral cytoplasm. No vacuoles, however, were present in Sudan black preparations, indicating that the vacuoles were probably produced by the largest cytoplasmic globules whose sudanophilia was observed by direct staining of cells under the microscope.

Since the standard Golgi stains failed to show any structures other than those which were revealed by supravital staining and by phase microscopy, it seems justifiable to consider the larger argentophilic and osmiophilic granules which resemble the sudanophilic, neutral red staining bodies as Golgi bodies or lipochondria (38).

In smear preparations of tumor cells (but not paraffin sections) fixed in formol sublimate and stained with dilute toluidine blue, there were numerous dark blue cytoplasmic granules, identical in shape, size, and distribution with the supravitaly stained mitochondria (Fig. 1).

In summary, therefore, at least three types of sudanophilic material were demonstrated in the cytoplasm of the tumor cells. The smallest and most numerous particles were mitochondria; a few larger granules, mainly in the nuclear hof, were lipochondria or Golgi bodies; and, until they can be more clearly defined, the largest will be called lipid globules.

Cytochemistry.—The tinctorial and cytochemical properties of the cytoplasmic and nuclear structures of the tumor cells are summarized in Table 2.

Nucleus and nucleolus.—The nuclear chromatin, including the nucleolar membrane, was the only material in the cell which gave a positive Feulgen reaction. In acid solutions of aniline blue and orange G the nucleolus and parachromatin granules had a stronger affinity for orange G than any other cell component, and therefore contained histone (42). The nucleolus was poorly stained by toluidine blue in smear preparations, and the intensity of staining was not decreased by ribonuclease digestion (Figs. 1 and 2). The nucleolus had the same staining range as the cytoplasm in the methylene blue extinction test, and was similarly affected by previous ribonuclease digestion (Table 2). The nucleolus, like the rest of the cell, stained dark red by the coupled tetrazonium reaction. Benzoylation eliminates the reaction of tyrosine, tryptophan, and histidine with this reagent, but, according to Danielli (10), has little effect on the reactivity of compounds containing purine and pyrimidine, which may not react at all under the conditions of this test (37). After benzoylation the nuclear desoxyribonucleic acid (DNA) was not stained by the tetrazonium method, but the cytoplasm was still faintly positive. Tyrosine, tryptophan, and/or histidine were therefore present in the nucleus, nucleolus, and cytoplasm. Extraction of the nucleic acids by treatment with sulfuric acid completely abolished the reactivity of all the cell constituents (Table 2). Ribonucleic acid (RNA) may have been responsible for the residual staining after benzoylation. The amount of RNA in the nucleolus and of DNA in the nucleus was not sufficient to react with the tetrazo compound after benzoylation. Confirmatory evidence of the paucity of RNA in the nucleolus was provided by the failure of ribonuclease to decrease the in-

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1 The term “nuclear hof” indicates the zone of cytoplasm which is the site of the centrosome and the lipochondria or Golgi apparatus and is usually adjacent to a slight indentation of the nucleus.
# Table 2

**Cytochemical and Tinctorial Properties of the RPL-12 Tumor Cell**

<table>
<thead>
<tr>
<th>Method</th>
<th>Barret’s stain</th>
<th>Feulgen</th>
<th>Lipid extraction</th>
<th>Nilesulphate Acid hematin</th>
<th>Sudan</th>
<th>Lipoextraction</th>
<th>Tetrasomic</th>
<th>Strong acid benzyoilation tetrasomic</th>
<th>Aniline blue, orange G</th>
<th>Acid phosphatase</th>
<th>Supravital staining</th>
<th>Probable constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatin</td>
<td>+</td>
<td></td>
<td>P.A.S.</td>
<td>Blue</td>
<td>Blue</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>Orange</td>
<td>0</td>
<td>Histone</td>
</tr>
<tr>
<td>Nucleolus</td>
<td>Blue</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>Pink</td>
<td>++</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>R.N.A. protein</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>Pink</td>
<td>++</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>Large lipid globules</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>Pink</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Glycolipid glycoprotein</td>
</tr>
<tr>
<td>Lipochondria</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>Pink</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Neutral lipid R.N.A. acid phosphatase</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>Pink</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Janus green</td>
</tr>
</tbody>
</table>

No detectable preformed aldehyde, acetal phosphate or aldehydes secondary to oxidation of unsaturated fatty acids, no birefringence, colored material, cholesterol, ascorbic acid, alkaline phosphatase or succinic dehydrogenase.

Par = Paraffin section  
F. sub. = Formal sublimate  
M.B.E. = Methylene blue extinction test  
Sudan black B or Sudan III and IV  
F. cal. = Formal calcium  
P.F.A.S. = Pericform acid Schiff  
Trace after prolonged incubation
tensity of staining with toluidine blue. Only the methylene blue extinction test provided any evidence for the presence of RNA in the nucleolus, and this may have been caused by the presence of this material in the cytoplasm, which almost invariably covered the nucleolus in sections of the thickness used here.

Lipid globules, lipochondria and mitochondria.—The cytochemical reactions of the sudanophilic cytoplasmic components (Fig. 4) may be conveniently considered together. All three components gave a pink color in all steps of Cain’s Nile blue sulfate method and therefore contained neutral lipid (8). In material fixed in formal calcium they all stained with Sudan III and IV, which suggests, but certainly does not prove, that they contained triglycerides (37). With or without fixation, the cytoplasmic particulates were colorless, monorrefrangent, and gave a negative Schultz reaction for cholesterol. No preformed aldehyde or acetal phosphate could be detected in unfixed smears. The performic acid Schiff test for unsaturated lipids was negative, and after 8 days’ exposure to air no oxidation aldehydes could be demonstrated. Phospholipid could not be demonstrated by Baker’s acid hematin test.

As already noted, the large cytoplasmic globules stained most readily with Sudan black B; they did not stain by any methods other than those involving simple solution of fat soluble dyes (Table 2) and probably consisted mainly of triglyceride.

In paraffin sections no P.A.S.-positive material could be demonstrated in the tumor cells. In smears stained by the P.A.S. method and mounted in glycerine jelly, a few irregularly sized P.A.S.-positive granules were observed in the nuclear hof. When such a preparation was counterstained with Sudan black, all the red stain was obliterated by the black. After extraction of lipids, slightly smaller, less intensely colored P.A.S.-positive granules could be demonstrated in the nuclear hof. There were, therefore, two P.A.S.-positive components associated with the sudanophilic granules of the nuclear hof which, as already shown, were the lipochondria. One component, which could be removed by treatment with hot chloroform and methanol, was a lipid; the other was resistant to treatment with lipid solvents.

Glycolipids, some phospholipids, and some unsaturated lipids are the only colorless lipids reported to give a positive P.A.S. reaction (48). The presence of phospholipids in any quantity was excluded by the negative acid hematin reaction and the failure to demonstrate acidic lipids by Cain’s Nile blue sulfate method (Table 2). Unsaturated lipid could not be detected by the performic acid Schiff reaction, and the reaction for aldehydes secondary to the oxidation of unsaturated fatty acids was negative (Table 2). Some reduction in the intensity of the P.A.S. reaction was produced by bromination, but no more than occurred in a lipid-free control section containing acid mucopolysaccharide. The reacting lipid was probably glycolipid.

The presence of acid mucopolysaccharide and glycogen in the lipochondria was excluded because the P.A.S.-positive reaction was unimpaired after hyaluronidase and diastase digestion and because metachromasia was absent. Acetylation in dry pyridine completely blocked the P.A.S. reaction of the cell, but, since pyridine is a lipid solvent, this did not provide any information about the reacting groups in the lipid component. It did, however, confirm that 1,2 glycol groups were probably responsible for the reaction of the other component which, by a process of exclusion, was either a glycoprotein or a neutral mucopolysaccharide.

In smears fixed in formol sublimate and stained with toluidine blue, the mitochondria were clearly stained dark blue (Fig. 1). The staining reaction must have been due to the presence of ribonucleic acid, since it was completely abolished by previous ribonuclease digestion (Fig. 2). The close association of RNA with the lipid particles was shown by the absence of mitochondrial staining with toluidine blue after extraction of lipid.

In unfixed and formalin-fixed preparations, the Gomori technic for acid phosphatase revealed minute positive granules throughout the cytoplasm of the tumor cells (Fig. 3), reaching a maximum intensity after 1 and 2 hours’ incubation, respectively. After this period some diffusion occurred, and the nuclear chromatin was also stained. The reaction was more intense in the unfixed than in the fixed material, but the number and distribution of the particles were identical and suggested that the enzyme was present in the mitochondria. In preparations fixed in acetone, staining appeared after 3 or 4 hours’ incubation and was more pronounced in the nucleus than in the cytoplasm.

Cytoplasm.—When paraffin sections were used, the mitochondria were no longer recognizable in toluidine blue preparations and the cytoplasm stained diffusely blue or faint purple. The staining reaction was removed by ribonuclease digestion, showing the presence of RNA. The presence of RNA in the cytoplasm was further confirmed by the alteration in the pH of extinction in the methylene blue extinction test after ribonuclease

1 P.A.S. = Periodic acid Schiff.
digestion (Table 2) and by the results of the tetrazonium reaction already described.

**Virus-infected Tumor Cells**

**Lipochondria.**—No perceptible changes occurred in the tumor cells until the 2d or 3d day after virus inoculation. At this time an increase in the number and size of the lipochondria (Fig. 5) could be demonstrated by all the methods described except supravital staining, which did not show any increase in the neutral red-staining particles. In some cells the enlarged lipochondria appeared to fuse to form one or two larger aggregations.

In paraffin sections, however, the early stages of the development of cytoplasmic aggregations were not seen, and the first abnormality in hematoxylin and eosin sections was the appearance of one or two large homogeneous eosinophil inclusion bodies in the nuclear hof. (The term “inclusion body” can be applied with some justification, since no such structure can be seen in similarly prepared sections of uninfected tumor cells.) These bodies stained uniformly red by the P.A.S. method, even after extraction of all stainable lipid. Like the Golgi bodies, their ability to stain with the P.A.S. technic was unaffected by diastase or hyaluronidase digestion, was reduced by bromination, and abolished by acetylation. They stained faintly with Sudan black B, and on high magnification sometimes appeared to consist of a number of smaller granules (Figs. 6 and 7). The “inclusions” differed from the smaller lipochondria of the uninfected tumor cell in that they were visible in paraffin sections, where they acquired properties which could not be shown in smear preparations of infected or uninfected tumor cells. In sections they stained more intensely than the cytoplasm, but exhibited the same staining reactions with the tetrazonium method, toluidine blue stain and the M.B.E. test, and were similarly altered by previous destruction of RNA by ribonuclease or treatment with strong acid. They contained, therefore, protein (amino acids) and RNA. Except for the presence of RNA and allowing for some loss of lipid in the imbedding process, the cytoplasmic “inclusions” observed in paraffin sections had the same cytochemical properties as the lipochondria of infected and uninfected cells in other preparations.

**Mitochondria.**—In supravital and smear preparations of virus-infected cells the mitochondria were unaltered in morphology and cytochemical properties. Since mitochondria were not demonstrable in paraffin sections of material fixed in formol sublimate, they may also have been involved in the fusion of lipid particles which gave rise to the “inclusions,” and in doing so imparted their RNA content to the final mass.

**Lipid globules.**—Coincident with the changes in the lipochondria, the cytoplasmic lipid globules were sometimes, but not always, increased in size and number.

**Nucleus.**—As the Golgi bodies increased in number and size the nucleus of the cell was pushed to one side and even indented by the larger aggregations (Fig. 7). Some margination of the nuclear chromatin was seen in an occasional tumor cell with hypertrophied Golgi bodies, but for the most part no further evidence of degeneration was detected until the cell had undergone phagocytosis. This latter process was extremely rapid when the tumor was growing in muscle, and tumor cells were no longer present within 2 days of the appearance of the first abnormality. In the brain, however, phagocytosis was delayed, and “inclusions” persisted in most of the tumor cells for 3 or 4 days, during which analysis of the mitotic process was possible.

**Mitotic process.**—From the results presented in Table 3 it will be seen that there was a significant increase in the percentage of tumor cells in mitosis after virus infection. Analysis of the data reveals that this was almost entirely the result of an increase of cells in metaphase. The percentage of infected tumor cells in the other stages of mitosis was decreased, though only the telophase results are significant. There was, therefore, a delay or partial arrest in metaphase. Further evidence of interference with the process of cell division was shown by a significant increase in the proportion of binucleate tumor cells and in the percentage of abnormal mitotic figures in the virus-infected series (Table 3). Clumping of the chromosomes in metaphase was the commonest abnormality of mitosis, but scattering of chromosomes and clumping in prophase, anaphase, and telophase were fairly frequent. The same types of abnormality were present in the uninfected and infected groups, and the difference between the two was entirely quantitative.

**DISCUSSION**

The results of this study lend support to the more recent views of Baker on the nature of Golgi bodies or lipochondria (3). The lipochondria of the tumor cell consisted of spherical bodies containing glycolipid, and some comprised an outer lipid layer and an inner zone of glycoprotein or neutral mucopolysaccharide. Cytochemical differentiation between glycoprotein and neutral mucopolysaccharide is impossible, but the co-
TABLE 3
ANALYSIS OF MITOSIS AND BINUCLEATE CELLS IN TUMOR AFTER INFECTION WITH N.F.T. VIRUS

<table>
<thead>
<tr>
<th>Day after inoculation of virus</th>
<th>Second</th>
<th>Third</th>
<th>Fourth</th>
<th>Fifth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of tumor cells in:</td>
<td>Uninfected</td>
<td>Virus-infected</td>
<td>Uninfected</td>
<td>Virus-infected</td>
</tr>
<tr>
<td>Prophase</td>
<td>2.2</td>
<td>2.2</td>
<td>1.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Metaphase</td>
<td>2.4</td>
<td>2.8</td>
<td>2.8</td>
<td>2.2</td>
</tr>
<tr>
<td>Anaphase</td>
<td>0.4</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Telophase</td>
<td>1.0</td>
<td>1.0</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Mitosis (total)</td>
<td>6.0</td>
<td>6.4</td>
<td>4.7</td>
<td>5.0</td>
</tr>
<tr>
<td>Abnormal mitosis (per cent of total mitosis)</td>
<td>5.3</td>
<td>6.2</td>
<td>8.8</td>
<td>2.0</td>
</tr>
</tbody>
</table>

| Binucleate cells (per cent of tumor cells) | 0.2 | 0.1 | 0.3 | 0.0 | 0.1 | 0.1 | 0.2 | 0.1 | 0.6 | 1.0 | 1.0 | 1.0 |

Means of observations:
- Uninfected: 1.843, 1.629
- Virus-infected: 1.843, 1.629

Standard error of difference between means:
- 0.214
- 0.1854

Difference between means:
- 0.004
- 0.4810
existent presence of protein suggested that the former was more probable. A similar conclusion was reached by Gersh (16), who demonstrated glycoprotein in the Golgi apparatus of the duodenal cells in the rabbit and guinea pig.

Supravital staining clearly demonstrated that in the infected tumor cells some of the lipochondria did not color with neutral red. Jackson (23) has recently reported a similar loss of affinity for neutral red affecting the granules of degenerating leukocytes.

The occasional presence of phospholipids in Golgi bodies has been described by Baker (8) and by Cain (9). Evidence for the existence of vitamin C and alkaline phosphatase in the Golgi bodies of many different cells has been reviewed by Bourne (7). These substances could not be demonstrated in the tumor cells (Table 2).

Hypertrophy of the Golgi bodies of virus-infected cells has been reported in canine distemper (11), Rift Valley fever (14), vaccinia (31), lopingill (21), myxomatosis (13), and fowlpox (32). The "inclusions" in the virus-infected tumor cells showed a remarkable resemblance to the Bollinger bodies of fowlpox. In a detailed study of the evolution of the Bollinger body, Ludford and Findlay (32) suggest that the elementary bodies of the virus are incorporated in a lipid mass derived from the Golgi bodies. Although the size of the N.F.T. virus is not known with certainty, the size of other strains of St. Louis encephalitis virus is probably 20–30 μ (12). Thus, there is little possibility of visualizing elementary bodies or even aggregations of particles of this virus with the light microscope. The only evidence for the presence of virus in the "inclusions" was indirect: viz., that the presence of "inclusions" in the cells coincided with the period when virus was demonstrable in maximal amounts in the tumor (30).

According to Palade and Porter (36) RNA is located in the endoplasmic reticulum of the cell. Differential centrifugation (24) and cytochemical studies (41) have shown that it is also present in the mitochondria. The present study indicates that RNA was present in diffuse and particulate form in the cytoplasm and was probably associated with the mitochondria. Support for the conclusion that acid phosphatase was present in the cytoplasm, and probably in the mitochondria, is provided by the differential centrifugation studies of Palade (38) and Berthet and de Duve (6). The latter found that 55 per cent of the acid phosphatase activity was associated with the mitochondria.

The nature of the large cytoplasmic lipid globules is obscure. Similar globules have been observed in the Ehrlich ascites tumor cell (28). Following the classical hypothesis that the Golgi bodies undergo a developmental cycle in relation to the secretion of the cell (3), it is tempting to speculate that the lipid globules represent the final stage in the process after the bodies have released their content of secretory material.

Although there are numerous reports of the inhibition of tumor growth by virus infection, as far as we are aware there is no record of viruses acting as antimitotic agents. Bunyamwera virus affected the intermitotic phase of the Ehrlich ascites tumor cell and had no effect on the various stages of the mitotic process (28). N.F.T. virus infection, on the other hand, was responsible for partial arrest in metaphase and some failure of cytoplasmic division. The cytoplasmic "inclusions" in the virus-infected tumor cells were of sufficient solidity to indent and displace the nucleus. The delay in metaphase might have resulted, therefore, from mechanical obstruction to the separation of the metaphase chromosomes.

SUMMARY

1. A cytological and cytochemical study of the RPL-12 tumor cell is presented. Three types of lipid particles were identified in the cytoplasm. The significance of the largest is unknown. The smallest were mitochondria and probably contained neutral lipid, RNA, and acid phosphatase. The remaining particles appeared to be lipochondria and contained glycolipid and glycoprotein.

2. Infection of the tumor with the N.F.T. strain of St. Louis encephalitis virus induced an increase in the number and size of the lipochondria, partial arrest of mitosis in metaphase, and increased numbers of mitotic abnormalities and of binucleate cells.

3. The mitochondria of the tumor cell were unaffected by virus infection, but appeared to fuse with the hypertrophied lipochondria in the process of paraffin imbedding and contributed to the formation of large cytoplasmic "inclusion bodies" seen in paraffin sections.

ADDENDUM

Duran-Reynals has observed that fowl pox was activated by painting the skin of chicks with methylcholanthrene (F. Duran-Reynals, Studies on the Combined Effects of Fowl Pox Virus and Methylcholanthrene in Chickens. Ann. New York Acad. Sc., 54:977–99, 1952). The similarity of the "inclusions" in the RPL-12 tumor cells infected with St. Louis virus to Bollinger bodies suggested that activation of latent fowl pox might have occurred. To investigate this possibility, a 10 per
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cent suspension of tumor, which had been infected 3 days previously with NFT virus and was known to contain "inclusion bodies," was inoculated in 0.2-ml amounts onto the chorio-allantoic membranes of ten 7-day-old chick embryos. Two embryos were harvested daily, and no gross or histological evidence of fowl pox was observed.

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Studies on a Transplantable Chicken Tumor (RPL-12 Lymphoma): III. Cytological Changes during Virus-induced Oncolysis

Robert Love and George R. Sharpless


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