Summary

A procedure for obtaining a high percentage of Rous sarcoma virus-infected cells in cultures is described. Approximately 50% of the cells were infected by the 3rd day, as determined by infective center assay. Virus assayed 24 hr after infection showed only 10^6 infective particles/ml of supernatant fluid. Maximal titers ranged between 10^8 and 10^9 infective particles/ml from the 5th day on. Cultures from special embryos from a flock free of lymphomatosis were found to give slightly larger numbers of infected cells than those from the open flock.

Periodic acid-Schiff-positive, diastase-resistant material was found in the cytoplasm of converted cells in the paranuclear area and also close to the cell surface. None of this material was found at the cell membrane. An increased amount of staining with reduced diphenolphosphorylene nucleotide-nitro-blue tetrazolium (NBT) and succinate-NBT systems localized in a pattern similar to mitochondria was found in converted cells. Larger amounts of acid phosphatases were found as well in converted cells.

Introduction

Cytologic studies of cells in vitro, infected with Rous sarcoma virus (RSV), have been conducted from different points of view by several investigators (11, 28, 29). In general, the growth of RSV induces the formation of altered cells (19, 20). Rous sarcoma cells show large nuclei and basophilic cytoplasm (26). Their movements are not restricted by contact inhibition, and multinucleated cells eventually develop. Biochemically, there is an increase in lactic acid production and infected cultures show the Crabtree effect (18). These characteristics are also present in Rous sarcomas produced by inoculation of cell-free preparations on the chorioallantoic membrane (CAM) (30).

Cytochemically, Erichsen et al. (10) demonstrated the presence of mucopolysaccharides in chick embryo cells infected with RSV. This agrees with Kabat (17), who studied both avian leukemia and sarcoma of fowl. Hampton and Eidinoff (12), by using tetrazolium salts, showed a greater precipitation of the dye on RSV-infected cells in a test involving the lactic dehydrogenase-nitro-blue tetrazolium-reductase complex, but no differences were found when mitochondria were stained with Janus green B.

The development of technics which use tetrazolium salts together with various substrates, and result in a cytochemical reaction that is disclosed by a colored formazan precipitate in the specific sites of enzyme activity, permits the demonstration of several different substrates and enzymes. This paper attempts to relate the morphologic changes, the growth of virus, and several cytochemical changes in RSV-infected chick embryo cultures.

Materials and Methods

Virus

A chick embryo passage of the Bryan strain of RSV was used. The virus had been cloned by 2 passages of i.v. inoculation in special chicken embryos from an isolated flock maintained by the department (1). This flock has no antibodies to Rous virus and is assumed to be free of virus. Livers showing vascular lesions were used for the subsequent passages. Such virus suspension and embryos are referred to hereafter as cloned vascular antibody-free (CVA) strain and antibody-free (AF) embryos, respectively. Embryos obtained commercially, in which the parental presence of antibodies against RSV was frequently present, are referred to as open flock (OF) embryos. The CVA strain received at the 10th passage was further inoculated on the CAM of 12-day-old AF embryos, and the tumors which developed were harvested and used to prepare a 20% w/v suspension which was then centrifuged at 2500 rpm for 15 min at 2°C. This preparation had a titer of 4.2 × 10^6 viral lesion-forming units (VLFU)/ml, as determined by i.v. inoculation of 10-day-old AF embryos. Virus suspension and its dilutions were prepared with citrate buffer, pH 6.8. From the 20% suspension, 2-ml aliquots were placed in ampules and stored at -30°C.

Cell Cultures

Cell suspensions from six 10- to 12-day-old OF and AF embryos were separately prepared and washed with Hanks’ balanced salt solution (Baltimore Biological Laboratories) 3 times after trypsinization, and in each case were divided into 3 groups each.

Group I. Chicken cells were placed in fresh medium and cultured in plastic bottles (purchased from Falcon) and on cover slips in Leighton tubes. Cover slips were removed every 24 hr for staining. Cells from the bottles were used to prepare other cultures on cover slips when needed. Some bottles were used as a
monolayer on which to plate infected cells for assay of infective centers (26). When these foci were counted, satellite foci were not included, since these may arise secondarily to the original foci despite the presence of antisera.

Tumors were induced in 4-week-old chickens by inoculating i.m. in the breast muscle 1.0 ml of a 10^7 suspension of RSV. Chickens which developed regressing tumors were selected and infected once more with the same dose by the same route. Sixteen days after the 1st inoculation, chickens were bled and individual sera assayed for RSV antibodies by mixing a 1:10 serum dilution with equal parts of 200 VLFU of RSV in such a way that the inoculum (0.05 ml) contained 100 VLFU and final serum dilution was adjusted to 1:20. The mixture was then inoculated i.v. into 11-day-old chicken embryos. Sera which inhibited at least 85% of hemorrhagic lesions, as compared with controls (chick embryos inoculated i.v. with 0.05 ml of suspension containing 100 VLFU), were pooled for further use as indicated.

**GROUP II.** Immediately after washing, a heat-inactivated virus suspension or a suspension of normal CAM from AF embryos was added to this cell suspension. The combinations were incubated at 38°C for 1 hr and were frequently stirred. The cells were then washed 3 times by centrifugation at 600 rpm for 10 min, resuspended in Hanks' solution, and then cultured as those from Group 1.

**GROUP III.** Cell pellets containing approximately 10^7 cells each were suspended in aliquots of 2 ml of active virus suspension containing 4.2 × 10^7 infective RSV particles/ml. Cells and active virus were incubated and washed in the same manner as those from Group II, with 10 ml Hanks' solution. No free virus was detected in the supernatant fluid. Part of the cells from the cover slips were resuspended in chicken anti-RSV sera after incubation with 0.1% trypsin. The cells were then plated on uninfected monolayers of AF embryo cells to determine infective center assays.

The medium was commercial tissue culture solution 199, calf serum, and tryptose phosphate broth (26). Cells from AF and OF embryos were always kept separately. An additional set of cultures in which the pH was carefully controlled at 6.8 by changing the medium twice a day was also prepared. They were otherwise treated identically with the above. Cover slips were removed at daily intervals and stained in most experiments.

**Infectivity Tests**

From control and infected cells of both AF and OF embryo cultures, fluid samples were taken every 24 hr, kept frozen at —30°C, and assayed for RSV by i.v. inoculation of 10- to 11-day-old AF embryos. Eight embryos were used at each dilution. After 6-7 days the entire embryo was harvested and all vascular lesions were counted in both the embryo and its membranes. The titers were determined from the average number of lesions in each dilution (6).

On the 3rd, 5th, and 7th days, cells from the cultures were trypsinized (0.1% trypsin) and washed 3 times with Hanks' solution by repeated centrifugation. Cells were counted in a hemocytometer, and aliquots containing approximately 100 viable cells were plated over AF embryo monolayers. The cells were allowed to attach by incubating for 6 hr in a small amount of medium, and were then covered with agar to which specific antibody was added. On the 6th day, fresh medium was added to the agar overlay, and on the 10th day, cell foci were counted (26).

The use of primary cells was developed, since it was difficult to obtain a sufficiently high proportion of infected cells by inoculating secondary cultures in monolayers as recommended by Rubin (26). Later on, it was found that by placing the cell pellets with a small amount of fresh medium in the refrigerator (2°-4°C) for 1-2 hr before the challenge, the percentage of infected cells increased further, and this modification was also added to the procedure.

**Staining Procedures**

For general morphologic observations, the cells were stained with 1.0%, aqueous toluidine blue, after fixation in absolute ethanol for 24 hr. After staining, the cells were washed in tap water and air-dried, immersed in acetone for 5 min, cleared in xylene, and mounted in Permount.

**ACID PHOSPHATASES.** After fixation in 10% neutral formaldehyde at 4°C for 6 hr, cells were stained as follows: Control and infected cultures were placed in Coplin jars, and 0.85% saline was added. After 5 min the cells were washed thoroughly, 1st in 1.0% saline and then in 2.0% saline solutions. They were incubated for 2 hr at 37°C in a solution of sodium 6-benzoyl-2-naphthyl phosphate in citrate buffer, pH 5.0, and washed afterward in normal saline. By using fast red in distilled water (1 mg/ml), a postcoupling reaction was obtained at the sites of enzyme activity. The cells were counterstained with fast green (0.1% in absolute ethanol), and after being washed in water were mounted in polyvinyl pyrrolidone (7).

**MITOCHONDRIAL STAINING.** Since no morphologic alterations have been shown to occur in the mitochondria of RSV-infected cells by cytochemical means and no mention is found as to whether or not biochemical changes occur at this level, it was decided to investigate mitochondrial activity by the use of tetrazolium salts (23). After fixation for 10 min in calcium formamide, the cells were incubated in phosphate buffer (pH 7.4) containing 5 mg/ml of the reduced form of diphosphopyridine nucleotide (DPNH) and 1 mg/ml of nitro-blue tetrazolium (NBT). The incubation was maintained at 37°C for 25-35 min. Then the cells were washed in distilled water, counterstained very lightly with pyronin (pH 6.0), dehydrated, and mounted in Permount. The same procedure, but with sodium succinate as substrate, according to the method of Nachlas et al. (21) as modified by Rosa and Kwan-Chung (25), was used to stain another set of cover slips with cells for the demonstration of succinic dehydrogenase.

**OTHER STAINING PROCEDURES.** Esterases were studied by means of the Shinitka and Seligman method (27) with sodium fluoride as an inhibitor, and the cultures were stained with both fast red for inhibitor-resistant and fast blue for inhibitor-sensitive esterases. After fixation in absolute ethanol at —20°C for 48 hr, cells were stained with the periodic acid-Schiff (PAS) technique; oxidation for 1 hr was accomplished with 0.5% alcoholic periodic solution. One-hr digestion at 37°C with malt diastase before staining with PAS was performed in a group of control and infected cells to rule out the presence of glycojen. Diastase solution (0.1%) was prepared in phosphate buffer, 0.02 M, containing 0.8% sodium chloride. Cells were counterstained with fast green.
Control and infected cultures were always stained at the same time in the same Coplin jar.

Direct Observations

Large cover slips (45 × 53 mm) were placed in Petri dishes, and a cell suspension previously infected with RSV was seeded on them. Controls with or without heat killed virus were prepared at the same time. Once the cells were properly attached to the glass surface of the cover slip, the cover slip and the cells on it were placed over the 1.5-inch hole of a metal plate. A drop of fresh medium was added to the cells, and the other side of the hole was covered with a clean sterile cover slip (G. O. Gey, personal communication). The preparation was sealed with paraffin and studied in a phase contrast microscope inside an incubator at 38°C. Continuous observations of these cultures were made, and a written record of cell behavior was kept. The mitotic index was calculated in control and infected culture, counting 1000 cells in each slide and calculating the average. Mitoses were counted at the 2nd, 4th, and 6th days.

Filters were used for photomicrography in order to diminish the background, red or green according to the stain used (pyronin or fast green).

In order to make unbiased interpretations, labels on individual slides were covered with adhesive tape and numbered at random. Tabulation of findings was done without knowledge of the history of the culture.

Results

Sequential histochemical studies on tissue cultures of virus-infected cells are difficult unless a high proportion of the cells have been infected at about the same time. On the first day after incubation, 35% of AF and 30% of the cells in OF cultures were infected. By 3 days, 40-65% of the AF cells were infected when virus had been added immediately after trypsinization and refrigeration. In cultures from OF embryos, between 25 and 47% of the cells were infected.

By the 7th day, 100% of the cells were infected in cultures from AF embryos (Chart 1), but only 72% were infected by the 9th day in cultures from OF embryos (Chart 2).

In general, after the cells were infected, the pH in the fluid medium dropped faster than in the control cultures. In both, however, the medium was changed every 2 days. By the 2nd day, measurable amounts of extracellular virus \((10^4)\) were obtained from the fluid media of the infected cultures. This titered \(10^4\) to \(10^6\) VLFU/ml on the 5th–7th day. A decrease in virus concentration was noticed when the cells were transferred from the bottles to prepare cultures over cover slips, but returned to the previous level 2 days later.

Cell cultures infected with RSV for phase contrast microscopy were studied 5 times each day at 2-hr intervals for 7 days (24 preparations). Early changes included an increase in refringency of the rounded cells and thickening of the cell membrane. Four or 5 days after infection the altered cells became very active and showed ameboid movements. A mass developed in the paranuclear zone; lobulations and budding protrusions extended toward neighboring cells. Subsequently many thick-walled vacuoles formed in the cytoplasm. On occasions when 2 cells attached to each other, they seemed to exchange cytoplasmic material. Later they separated again. In other instances, they remained united, and eventually a large syncytium was formed.

Other cells which were not as active formed buds or droplike formations which were less refringent than the cell surface. These formations were 0.5–2 µ in their long axis, rather numerous and distributed all around the cytoplasmic membrane (Fig. 1). They eventually detached from the cell and burst. Eventually a multinucleated cell, 50 µ or more in diameter, was formed which contained 15 or 20 nuclei irregularly distributed around a mass containing particulate formations (Fig. 2).

Control cells remained stellate; the cytoplasm was clear and contained few small vacuoles. The cytoplasmic membrane was thin and well spread. The nuclei were round or oval and had a well-defined membrane, and the nucleoli were well differentiated. Rarely, small, round cells were present among the fibroblast-like cells. The mitotic index showed no significant difference from that of the infected cultures (Table 1).

Large multinucleated cells present in the infected cultures had granular and refringent mitochondria, whereas mitochondria in the control cultures appeared thin and filamentous. The cells in the middle of a focus were loosely attached to the neighboring cells, but those on the periphery of the focus were firmly attached to the glass surface.

**Chart 1.**—Virus titer and percent infected cells in cultures from antibody-free (AF) embryos. This chart represents an average of 6 experiments.
**Chart 2.**—Virus titer and percent infected cells in cultures from open flock (OF) embryos. This chart represents an average of 6 experiments.

**Cytochemical Changes**

Six slides in each of 12 experiments were stained for the demonstration of acid phosphatases. Uninfected cultures showed a fine, particulate distribution of orange-red material throughout the cytoplasm (Fig. 3). Infected cultures showed large red granules inside vacuoles along with a particulate distribution in the cytoplasm (Fig. 4). The latter were somewhat larger than those of the controls. The staining became more intense at the time of peak virus production (5th day), decreased by the 6th or 7th day, and, in older cultures, remained at a low level, but always more than in control cultures of the same age. Preparations counterstained with fast green showed some of the alterations seen in phase microscopy, in particular the paranuclear mass. Precipitates or nuclear staining which may be found by using Gomori’s method for acid phosphatase were not found with Burstone’s technic (8). The above changes were consistently found.

Staining of mitochondria with either DPNH or succinate as substrates and NBT as indicator were highly satisfactory. Formazan deposits were found (96 slides) in infected cultures, irregularly distributed throughout the cytoplasm and also densely accumulated on top of the paranuclear basophilic masses (Fig. 5). In these lightly stained cultures the intensity of the stain was greater in converted cells (dark blue or almost black). Some of the larger cells showed a pale halo around the nuclei when succinate was used (Fig. 6). Some cells believed to be infected with RSV had filamentous structures in the cytoplasm (mitochondria?), with dark formazan deposits over them. Control cultures, on the other hand, showed filamentous structures, also presumably mitochondria, regularly distributed in the cytoplasm with blue deposits over them, but lighter in color than those from infected cells. When succinate was used, control cultures were not stained as darkly as with DPNH-NBT. Such mitochondria were also found in infected cells stained by formazan where they were irregularly distributed, but massed in the paranuclear area.

Esterase activity was not usually demonstrated in either the infected or control cultures even when long incubation periods (up to 6 hr) were employed. Shortly after culturing the cells (12 hr), however, some activity was found in a few scattered cells in both control and infected cultures.

Staining with the PAS technic showed diastase-resistant material in various localizations in converted cells (66 slides). During the first 3 days after infection the PAS-positive material was present in small amounts in the cytoplasm of converted cells close to the nucleus or in infected fibroblasts which did not show any sign of conversion except the hypertrophied paranuclear mass. The PAS-positive material was distributed in small inclusion-like bodies within the paranuclear mass. These round bodies increased in size on the 4th day, and from the 5th day on (at peak extracellular virus titers) appeared at the periphery of the cell (Fig. 7). Near the cytoplasmic membrane they accumulated in irregular deposits (Fig. 8). None of the PAS-positive, diastase-resistant material was found in the control cultures (Fig. 9).

**Discussion**

It should be pointed out that the Bryan strain of RSV which was used in these experiments is thought to need a lymphomatosis virus in order to produce new virus. In other words, it is a defective strain of virus (13, 14). Thus it is not known which of the observed changes may be due to lymphomatosis and which to RSV.

Under the conditions of these experiments, an increase in acid phosphatase seemed to be demonstrated in RSV-converted cells, as shown by the presence of large stained granules inside vacuoles. This may also be considered as part of the lysosome system (9), a localization of enzyme activity rather than an increase. Four different acid phosphatases have been isolated from rat liver (2, 3), but it is not known whether one or several of these or an entirely different acid phosphatase is the one here studied. It should also be pointed out that fixation of cells with neutral

---

**Table 1**

<p>| MITOTIC INDEX IN ANTIBODY-FREE (AF) EMBRYO CELL CULTURES (1000 cell counts/slide) |</p>
<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

---

**Francisco Salido-Rengell**
formalin may account for inhibition of some acid phosphatases, thus leading to a more distinct staining of the remaining enzymes, resistant to the action of the fixative. The sodium 6-benzoyl-2-naphthyl phosphate in combination with diazonium salts used here is the best substance available for the demonstration of acid phosphatases.

Of special interest in the present experiments is the histochemical demonstration of oxidative enzymes, particularly the succinic dehydrogenase and the DPN-linked dehydrogenases. It must be remembered that the tetrazolium salt utilized here (nitro-blue tetrazolium), is one with a high oxidation-reduction potential which was increased by the pH at which the reaction was carried out (pH 7.4). The penetration of the NBT was also satisfactory after fixation of cells at low temperature, as shown by the consistent deposition of formazan microprecipitates on structures presumed to be mitochondria. It is possible that the increased staining in RSV-converted cells is due to altered permeability of mitochondria.

With the succinic dehydrogenase stain, presumably the hydrogen ions are transferred by a carrier, since tetrazolium salts are not known to accept electrons directly from the dehydrogenases. Therefore, the resulting staining (formazan deposits) may indicate the localization of the enzyme. The histochemical demonstration by Barnett et al. (4, 5) that succinic dehydrogenase is localized in mitochondria, indeed on the cristae, is further evidence that mitochondria are here stained, but the possibility of formazan deposits on lipid droplets must be considered (22).

In the cytochemical demonstration of the DPN-dependent dehydrogenases as well, the tetrazolium salt (NBT) is the electron acceptor. In fixed cultured cells, incubated with DPNH in the presence of NBT, a large amount of formazan deposit is obtained. Since DPNH does not directly reduce the tetrazolium salt, the reduction is carried out by flavoproteins.

At least 9 dehydrogenases of the DPN-DPNH system demonstrated by tetrazolium salts are present in cultured cells. Some of them are malic dehydrogenase, glutamic acid dehydrogenase, lactic acid dehydrogenase, alcohohlic dehydrogenase, glycero-phosphate dehydrogenase, and isocitric dehydrogenase (15, 16, 24). Any or all of them may be present in RSV-converted cells. Thus, the citric acid cycle may also be affected by Rous infections. However, it may be pointed out that the sensitivity of dehydrogenases to manipulation prior to the incubation of cells in the DPNH-tetrazolium medium, the optimal medium for such incubation, and the kinetics of the tetrazolium salts reactions are not yet fully analyzed. Formazan granules were deposited after incubation of cells in the DPNH-tetrazolium medium, the optimal medium for such incubation, and the kinetics of the tetrazolium salts reactions are not yet fully analyzed. Formazan granules were deposited after incubation in DPNH-NBT in arrangements similar in morphology and localization to those of mitochondria, which suggests that the lavish network of formazan deposits shown in RSV-converted cells are formed by mitochondria.

The finding of PAS-positive material which is resistant to digestion with diastase is important. This may be due to several things; mucoprotein, glycoprotein, glycolipids, sphingolipids, or neutral mucopolysaccharides. The presence of acid mucopolysaccharides on the surface of RSV-converted cells has been found previously (10, 17), and Erichsen et al. (10) suggested that this may be considered as evidence of the conversion of normal cultured cells into Rous sarcoma cells. Whether or not there is a biochemical relationship between these acid mucopolysaccharides and the above mentioned PAS-positive material is not known.

Rubin (26) states that only 10% of the cells of an infected suspension develop foci immediately after infection, and he recommended the use of secondary cultures in monolayers to obtain a higher efficiency of infection. It has been shown here that primary cultures may eventually yield a high percentage of infected cells. Furthermore, it has been shown here that when primary cell suspensions were placed for 1 or 2 hr at 4°C immediately after trypsinization and were subsequently infected with high doses of RSV, the percentage of infection was improved by the 3rd day, yielding at least 50% of infected cells/culture.

Several factors may contribute to the success in obtaining a high percentage of infected cells. Among these are the facts that (a) suspended cells offer more of their surface for virus attachment; and (b) refrigeration of suspended cells has been used to induce a lag period in cellular metabolism. When the cells are returned to 38°C, cell metabolism and pinocytosis presumably proceed at a faster and more synchronized rate; thus, attached virus might penetrate into the cell more easily.

The cytochemical findings may be related to each other in that the RSV-induced cell conversion may be accompanied by increased enzyme activity, and the production of mucoproteins. An RSV-converted cell has an altered metabolism and is synthesizing proteins for virus capsids and for its own requirements. Acid phosphatases are related to protein synthesis, since it has been shown that large amounts of these enzymes are present in cells with high protein production, e.g., endocrine gland cells. Acid phosphatases are also related to the synthesis of S-S proteins, and PAS-positive material here demonstrated might be constituted by mucoproteins of the S-S protein type.

The fact that mitochondria are present in larger numbers and that there may be an increase in oxidative phosphorylation may indicate that converted cells have high energy requirements. Thus, there is “hypertrophy” of mitochondria.

Acknowledgment

The author is grateful to Dr. Frederik B. Bang for his valuable assistance in the preparation of this manuscript and his judicious advice.

References


FIG. 1.—Chick embryo cell, 24 hr after infection. Arrows indicate membrane evaginations containing protein and nucleic acid.

Fig. 2.—Converted multinucleated cell. At the periphery the dark area stains basophilically like protein and/or nucleic acid.

Fig. 3.—Control culture stained for acid phosphatases. Notice the particulate distribution of the enzyme.

Fig. 4.—Converted chick embryo cell (4th day after infection with Rous sarcoma virus). Dark dots indicated by arrows are acid phosphatase-stained material.

Fig. 5.—Mitochondria in a converted cell with a lavish appearance (arrow) after staining with reduced diphosphopyridine nucleotide (DPNH)-nitro-blue tetrazolium (NBT).

Fig. 6.—Three converted cells after succinate-NBT staining. The arrow points to a normal-looking fibroblast.

Fig. 7.—Cell after periodic acid-Schiff (PAS) staining. Arrows point to the PAS-positive, diastase-resistant material.

Fig. 8.—Same as in Fig. 7, but mucoprotein is accumulated at the peripheri in large clumps.

Fig. 9.—Control after PAS and diastase treatment. Dark staining is due to fast green staining. No PAS-positive material was present.
Cancer Research


Cytochemical Studies in Cell Cultures Infected with Rous Sarcoma Virus

Francisco Salido-Rengell

Cancer Res 1966;26:1031-1037.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/26/5/1031

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.