COMPARATIVE STUDIES IN ROUS SARCOMA WITH VIRUS, TUMOR CELLS, AND CHICK EMBRYO CELLS TRANSFORMED IN VITRO BY VIRUS*

I. Production of Mucopolysaccharides

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Infection of chick embryo or chick fibroblasts *in vitro* with Rous sarcoma virus is associated with the quantitative formation of foci of altered cells (1, 2) which continue to multiply and release virus (3, 4). This morphologically new and stable cell type with its enlarged nucleus, prominent nucleolus, and increased cytoplasmic basophilia (5) closely resembles the malignant cell seen at the center of tumors induced in chicks by injection of Rous sarcoma virus (6). Furthermore, *in vitro* these cells exhibit certain characteristic properties of cancer cells *in vivo*, in that they rapidly produce lactic acid (1, 2, 5) and are not restricted in their growth by contact inhibition (5). It was of interest, therefore, to determine if such cells would elaborate *in vitro* the mucopolysaccharide which appears between the tumor cells *in vivo* (6) and is responsible for the mucinous character of the myxomatous Rous sarcomas. This would provide evidence that chick embryo fibroblasts infected with Rous sarcoma virus *in vitro* are analogous to typical Rous sarcoma tumor cells *in vivo* in their synthetic as well as their morphologic, metabolic, and proliferative properties.

Materials and Methods

Virus.—The Rous sarcoma virus (strain A) stocks used were partially purified preparations kindly supplied by Dr. W. R. Bryan of the National Cancer Institute (5), containing 10^5 to 10^6 pock-forming units (PFU) per ml, as determined by counts of tumors produced on chorioallantoic membranes of a highly susceptible strain of chick embryos.

Tumors.—For production of tumors for histological or tissue culture studies, the stock virus was diluted to contain 2000 PFU per ml, and 0.1 ml (200 PFU) was injected into the wing web of 2- to 4-week-old white leghorn chicks. Tumors were removed for use when they were about 1 to 2 cm in diameter.

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Tissue Cultures.—

Tumor cells: The tumor tissue was removed from the wing web by aseptic dissection and minced with scalpels. The fragments were rinsed several times with Hanks's balanced salt solution (HBSS) in a centrifuge tube and then transferred to a flask containing 20 ml of 0.25 per cent trypsin (Difco) 1:250) in HBSS and a few drops of hyaluronidase (Rondase-Evans). After 10 minutes at 37°C, the supernatant was removed and new trypsin added. The first bloody trypsin solution was discarded, but the second, third, and fourth supernatant fluids were pooled in an ice bath and spun down at 2000 RPM. to recover the cells. These sedimented cells were resuspended in Eagle's medium (7) containing 20 per cent bovine fetal serum and distributed to Roux bottles for growth. When a good cell layer had developed, the cells were washed with HBSS, covered with 0.05 per cent trypsin, removed from the bottles, and sedimented with the centrifuge. The cells were resuspended in Eagle's medium containing 5 per cent bovine fetal serum and 500,000 cells in 2 ml of medium were added to tubes containing a coverslip for cultivation and incubated at 37°C.

Chick embryo cells: 10-day-old chick embryos from a suitable strain of susceptible chickens were used to prepare primary cultures by the methods described (3). When the cell layers had grown out on Petri dishes in a CO_2-air flow incubator, they were harvested with 0.05 per cent trypsin, sedimented, resuspended in Rubin's medium (3) containing 5 per cent calf serum, and 200,000 cells in 2 ml inoculated into a test tube containing a coverslip for cultivation. After 3 days of incubation at 37°C, the cultures were infected with 200 PFU of the Rous sarcoma virus.

Staining.—The tumor tissues were fixed in neutral buffered formalin, embedded in paraffin, and sections cut and mounted for staining. The coverslips with tumor cells or chick embryo fibroblasts infected with Rous sarcoma virus were fixed in the same manner. All slides were stained with the PAS technique according to Hotchkiss (8), thionine according to Hale (9), alcian blue according to Mowry (10), and the colloidal iron technique of Rinehart and Abul-Haj (11).

Digestion experiments were performed with testicular hyaluronidase (Rondase-Evans) in a 0.1 per cent solution in phosphate buffer at pH 6.0. The tissue sections or coverslips were exposed to hyaluronidase at 37°C for 18 hours and controls were kept in phosphate buffer. After digestion, the sections and coverslips were stained as were the controls by the methods above.

RESULTS

Tumors.—In the Rous sarcoma tumor tissues there were large amounts of an intercellular substance easily stainable with the colloidal iron technique, giving a dark blue color. In the peripheral zone of infiltration of the tumor it was seen as fine strands between the tumor cells, but at the center of the lesion was present as large masses in which tumor cells were embedded (Fig. 1). Some of the tumor cells also showed such material within cytoplasmic vacuoles. Metastatic lesions in the same chicks also showed this material between the tumor cells infiltrating the liver parenchyma, but it was not seen in the uninvolved area of normal tissue. This intercellular substance stained metachromatically with thionine, light green-blue with alcian blue, but was PAS-negative. On treatment with hyaluronidase, the colloidal iron-staining material was removed from between the cells and from the cytoplasmic vacuoles.

Tumor Cells in Vitro.—When tumor cells were grown *in vitro* for as long as 15 days with subculture, a homogeneous, thread-like substance appeared be-

tween the cells, often enveloping them, which stained dark blue with colloidal iron (Fig. 3) and also gave staining reactions with thionine, alcian blue, and PAS similar to the intercellular material seen in the tumors. This material was removed by treatment with hyaluronidase (Fig. 4). The tumor cells also showed blue material within cytoplasmic vacuoles with the colloidal iron stain (Fig. 3), though it was not well stained with thionine or alcian blue. The cytoplasmic material was not removed with hyaluronidase (Fig. 4), which may be owing to a failure of the enzyme to penetrate these cells which were fixed *in situ*. The cultures of tumor cells showed a rapid production of acid requiring frequent changes of culture media.

Transformed Cells in Vitro.—Within 5 days after infection of the normal chick embryo fibroblasts with Rous sarcoma virus, the cultures showed multiple foci of typical transformed cells whose cytoplasm was filled with masses of small blue granules and often contained cytoplasmic vacuoles staining blue with colloidal iron. By the 9th day, blue staining material was also abundant between the transformed cells (Figs. 5, 7). The intercellular material also showed the characteristic staining reactions with thionine, alcian blue, and PAS. Following digestion with hyaluronidase, the intercellular material disappeared, but the intracellular material remained and stood out clearly (Figs. 6, 8). With the normal cells between foci in infected cultures (Fig. 5) or in the uninfected control cultures (Fig. 2), no blue staining material was seen between the cells with colloidal iron staining except in a few areas where heavy aggregates of cells occurred, and this material was PAS-positive in contrast to that seen in the foci of transformed cells which was PAS-negative. As the transformed foci appeared, there was a marked increase in the rate of acid production.

DISCUSSION

It has been shown that an intercellular substance—which is stained blue by colloidal iron, metachromatically with thionine, and light green-blue with alcian blue, is PAS-negative and is digested by hyaluronidase—is associated with sarcoma tumor cells *in vivo* and *in vitro* and with cells transformed by Rous sarcoma virus *in vitro*. By its staining reactions and digestion by hyaluronidase this material would appear to be an acid mucopolysaccharide belonging to the hyaluronic acid–chondroitin sulfuric acid group (12). This would appear to be the same material which has been studied in the tumors by histochemical methods (6) or isolated from tumors and subjected to chemical analysis by several investigators who found that the material was rich in hyaluronic acid (13) and contained sulfate-bearing polysaccharides (14). It thus appears that the chick fibroblast transformed *in vitro* by the virus acquires the property of synthesizing the characteristic mucopolysaccharides which are typical components of the tumor *in vivo* and are produced by tumor cells *in vitro*.

The possibility that the polysaccharides were carried over with the tumor

cells into the cultures from the tumor must be considered, but the frequent washing of cells with fluids containing hyaluronidase and the fact that with subculture and growth *in vitro* the production of polysaccharides continued, eliminates this contingency.

The relationship of the intracellular material to the extracellular polysaccharide is not entirely clear. It is probable that the intracellular material represents a collection of polysaccharide which is then secreted by the cell, because the staining reactions with colloidal iron are the same, and the material appears within the cells first. However, the difficulty experienced in staining the intracellular material with thionine and alcian blue, though it was PAS-negative, makes the histochemical data incomplete. The failure to remove this intracellular substance from the tumor cells or transformed cells *in vitro* with hyaluronidase makes it impossible to ascertain its content of hyaluronic acid which is a characteristic of the extracellular polysaccharide, but this is probably owing to the fact that these cells were fixed *in situ*, and therefore the cell wall was intact and not permeable to the hyaluronidase whereas cells in the sectioned tissues are cut open giving free access to the enzyme.

Infection with Rous sarcoma virus *in vitro* confers on the normal chick embryo fibroblast the capacity to synthesize a mucopolysaccharide characteristic of Rous sarcoma tumors and thus viral infection is associated with another stable change in the cell in addition to those described earlier (1, 5). It is possible that this represents a potential synthetic capacity of the normal cell which is suppressed until viral infection occurs but even under identical conditions *in vitro* the normal cells in uninfected cultures or even those normal cells adjacent to the transformed cell centers in infected cultures do not produce the mucopolysaccharide in quantities detectable by the methods employed. Intercellular material appeared in cultures of normal cells only where they formed heavy masses, and it was PAS-positive and thus of a different nature from the polysaccharide produced by cells infected with Rous sarcoma virus.

The morphologic change produced in chick embryo fibroblasts by this strain of Rous sarcoma virus (strain A of Bryan) is usually characterized by the appearance of round, basophilic cells as previously described (1, 2, 5) but it is important to note that lines may be cloned from this strain of virus which produce different morphological changes (15) and thus different genetic information may be contributed to the cell depending on the line of virus employed.

The chick cell transformed by Rous sarcoma virus *in vitro* thus acquires synthetic as well as certain morphologic, metabolic, and proliferative properties characteristic of Rous sarcoma tumor cells and malignant cells in general *in vivo* and *in vitro*. The transformation reaction may thus result in the formation of a stable cell truly analogous to malignant cells of the tumor *in vivo* and further studies are under way to investigate the properties of the transformed

cell *in vivo* to ascertain if it possesses the attributes of malignancy *per se* independent of its constant release of the tumor-inducing virus.

SUMMARY

The chick embryo fibroblast infected by Rous sarcoma virus *in vitro* acquires the capacity to produce the acid mucopolysaccharides which are found in the tumors caused by this virus and which are also produced by tumor cells *in vitro*. The transformed cell acquires synthetic as well as morphologic, metabolic, and proliferative properties characteristic of Rous sarcoma tumor cells *in vivo* and *in vitro* and the transformed cell may be analogous to the tumor cell produced by virus infection *in vivo*.

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EXPLANATION OF PLATE 44

FIGS. 1 to 8—Photomicrographs of tissue or tissue culture cells stained with colloidal iron. × 240.

FIG. 1. Primary tumor from chicken injected with Rous sarcoma virus.

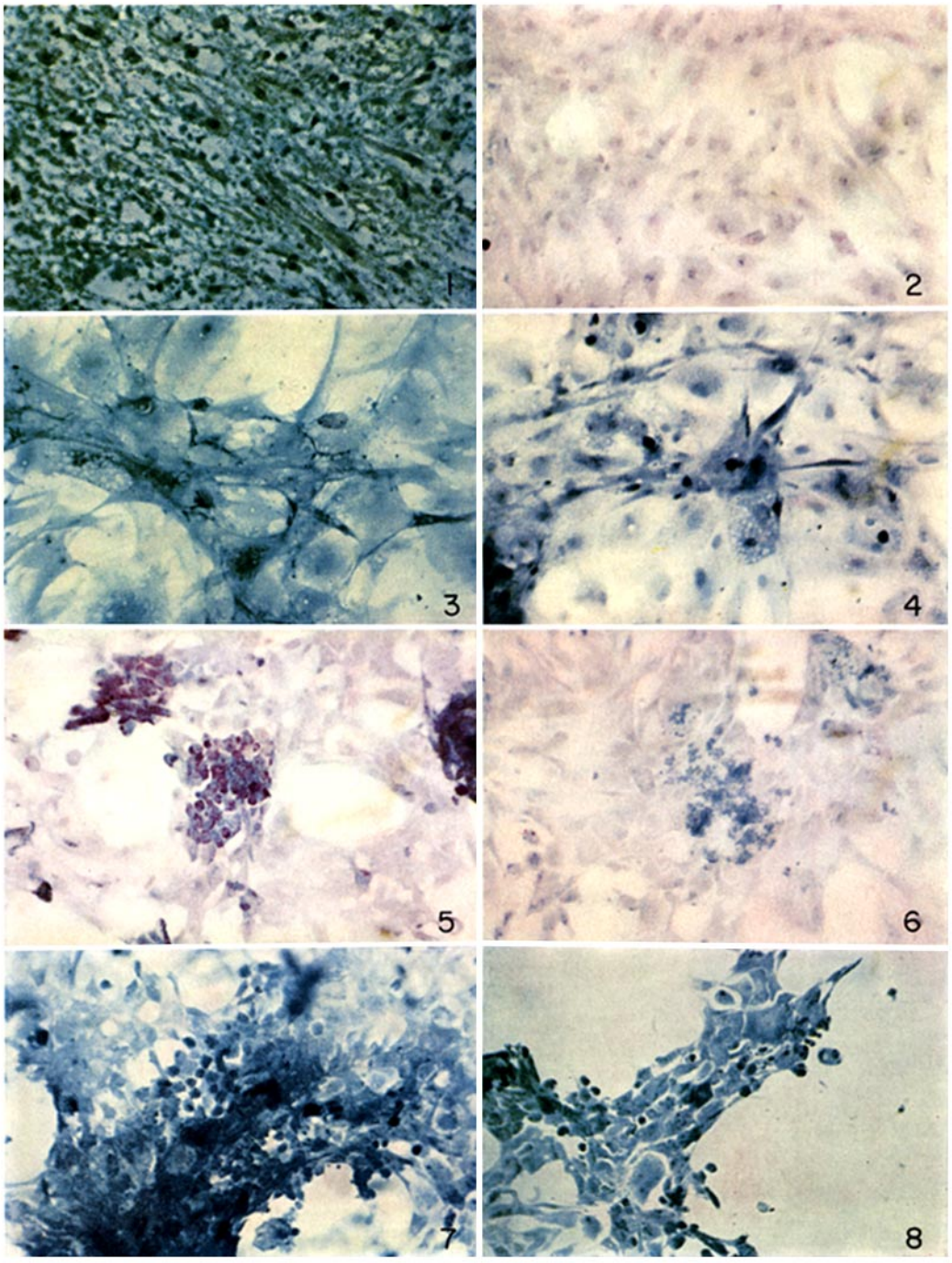
FIG. 2. Normal chick embryo cells in tissue culture.

FIG. 3. Rous sarcoma cells in tissue culture.

FIG. 4. Rous sarcoma cells in tissue culture after treatment with hyaluronidase.

FIGS. 5, 7. Chick embryo cells in tissue culture 9 days after infected with Rous sarcoma.

FIGS. 6, 8. Chick embryo cells in tissue culture infected with Rous sarcoma virus and treated with hyaluronidase.



(Erichsen *et al.*: Comparative studies in Rous sarcoma)