centration of NaCl in a medium with no calcium increased the cytoplasmic concentration of Na^+ to 8 m*M*. An equivalent amount of K^+ was lost in each case, and temperature changes did not affect the results. Chapman-Andresen and Dick (*12*) noted that the amoeba shrinks more rapidly during the first ten minutes of exposure to a hyperosmotic inducer solution than it does when exposed to a similar concentration of noninducer. Thus water permeability is probably increased in the low resistance state. Chapman-Andresen and Holter (*13*) showed that an amoeba is normally impermeable to glucose. However, external glucose is rapidly metabolized by the cells in the presence of an inducer of pinocytosis. Since the pinocytosis vacuoles are surrounded by a segment of plasma membrane internalized with the formation of the vacuole, these workers felt that the only explanation for their results was that there was a rapid change in the permeability of the membrane of the pinocytosis vacuole after it was incorporated into the cytoplasm. It is more probable that the membrane has a low resistance prior to its inclusion with the vacuole. In the experiment cited above, glucose could enter the amoeba cytoplasm by two paths: through the surface membrane and through the vacuole membrane.

The induction of a permeability change by stimulators of pinocytosis increases the flux of water, electrolytes, glucose, and molecules perhaps as large as ribonuclease (*14*) across the plasma membrane of the amoeba. The possible relationship of the induction of a permeability change by proteins in amoebae to the action of hormones such as insulin and antidiuretic hormones is discussed elsewhere (*15*). The similarity of pinocytosis to phagocytosis has been noted (*16*, *17*). The consequences of the surface attachment in phagocytosis leads to the same sequence of steps in uptake as that proposed for pinocytosis. In addition general changes in membrane permeability exist (*16*).

It is very provocative to treat the permeability changes induced in different cells by a variety of methods as having a general effect on solute penetration (*15*) rather than an effect on a single molecular species. A locus for change in membrane permeability seems indicated in amoebae by the similar effects on the membrane of two different classes of inducers of pinocytosis, the electrolytes and the proteins, and the all-important role of calcium. Even though these conclusions suggest that the membrane changes structure with a general increase or decrease in permeability, there is reason to believe that the relative permeability in lipids or water, the charge, and the size still regulate the relative rates of solute penetration. The change in permeability in the amoeba plasma membrane upon the induction of pinocytosis primarily involves a visible thickening of the lipid lamella evidently by reversible hydration of this element. Protein stabilization of the lipid lamella (*4*) must be contrasted with the capacity of some proteins to induce pinocytosis, apparently by the same mechanism as monovalent cations do. Stabilization of the amoeba membrane by calcium is a function of the relative concentration of monovalent cations, or appropriately charged proteins, and time. The methods and the results of this study may offer a new approach to the analysis of plasma membranes in general.

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Neoplastic Transformation of Rat Thymic Cells Induced in vitro by Gross Leukemia Virus

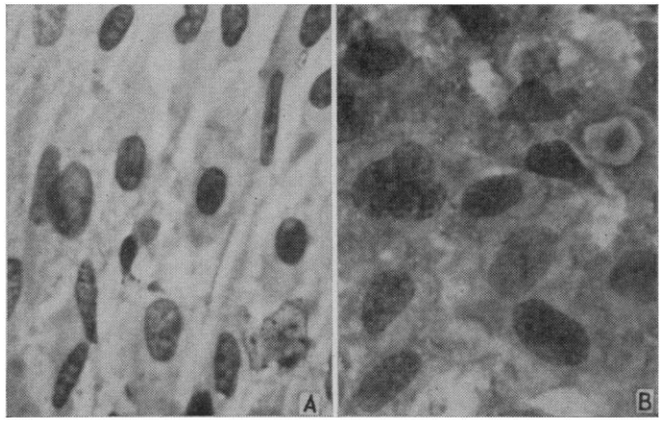
Abstract. *Cultures of embryonal rat thymus infected initially with Gross leukemia virus have, at the present time, abundantly replicated infectious virus particles for 20 months. Cells from these cultures, after 3 months in vitro, displayed morphological changes and induced formation of tumors upon isotransplantation. The tumors were serially transplantable, and subsequent transplants continue to carry the initial Gross leukemia virus.*

Gross leukemia virus (GLV) is the inducive agent of spontaneous leukemia in AkR mice (*1*) and has been adapted to produce a similar disease in the rat (*2*). It is, therefore, of interest to develop an in vitro system for studying both replication of this virus and virus-cell interrelationship. Moreover, tissue cultures in which GLV continuously replicates within supporting cells would provide a constant and reproducible source for virus.

Long-term replication of this virus in vitro has been achieved in cultures of both normal and leukemic rat thymus. In work reported previously (*3*, *4*), cultures of rat virus–induced thymomas were growing for 4 years, continuously replicating the initial GLV. In another system, normal rat thymic cultures, infected once at the beginning with GLV, have also been replicating the virus for long periods of time (20 months at present) (*4*). In both cases production of infectious virus particles has been demonstrated by means of electron microscopy and bioassays in susceptible animals.

Preliminary evidence indicates that cultures of thymic origin support and replicate GLV better than cultures of kidney and spleen (*4*). This report will deal with a particular event observed in these cultures, namely, that GLV is capable of inducing malignant transformation in long-term cultures of normal rat thymus.

Thymuses of normal W/Fu rat embryos were collected, minced, and suspended in Puck's medium. Gross leukemia virus from stock cell-free filtrates was added to the suspended cells;



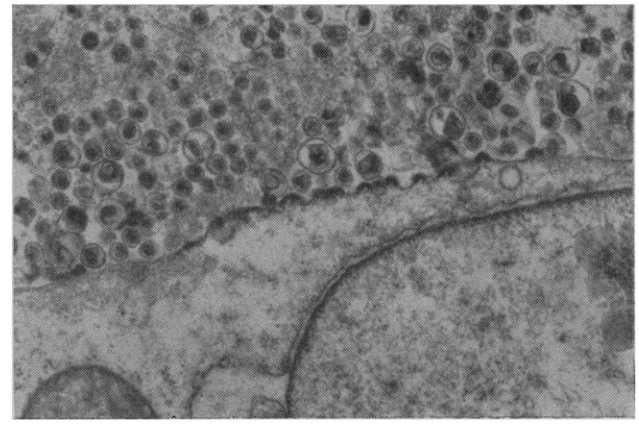


Fig. 1 (left). (A) Embryonal rat thymus in culture after 186 days. The epithelial-reticulum cells are regularly oriented; their nuclei are similar in shape, size, and staining characteristics. (B) Embryonal rat thymus in culture, 274 days after infection with GLV. Disoriented pattern of cellular growth; nuclei are irregular, coarse, and hyperchromatic (× 400). Fig. 2 (right). Electron micrograph of GLV-infected thymic culture after 285 days in vitro. Thymic cell displaying numerous virus particles budding at the plasma membrane (the appearance is that of semilunar electron-dense areas located along the plasma membrane situated at the midline of the picture). Large number of mature virus particles are in the extracellular space (× 32,000).

and cells and virus were mixed on a shaker for 1 hour at 4°C. The thymic cells were then deposited in plasma drops and placed in petri dishes. Monolayer cultures were obtained at the end of the first month and were then periodically trypsinized and subcultured. Similar procedures were used for uninfected control cultures.

Embryonal rat thymus in culture displays a particular morphology that can be studied in Giemsa-stained preparations on cover slips. After disappearance of the lymphocytes, which die out in the first few days, the pattern is that of a network of large, spindle-shaped, sometimes epithelioid, cells. In some areas these cells have polyhedral shapes and display pavemental arrangements. Presumably, their origin is the "epithelial-reticulum" of the thymus.

After about 3 months, morphological differences were noted between GLV-infected and control cultures (Fig. 1). The infected cultures grew more abundantly and required shorter intervals between subculturing. Piling-up and criss-cross growth of cells, although seen occasionally in normal cultures, appeared more frequently in the infected counterparts. The epithelioid pattern was more common, and large cells with atypical nuclei, sometimes multinucleated, were noted.

At this time, electron micrographs (*5*) displayed a large number of virus particles in the GLV-infected cultures. Mature virions, as well as immature forms budding at the plasma membrane, were observed (Fig. 2). Bioassays in mice and rats were positive, up to 100 percent, after an average latency period of 90 days. These periodically performed tests, up to 20 months at the present time, have all been positive. A full survey of the replication of GLV in these cultures, including the ultrastructural aspects, is reported elsewhere (*6*).

Starting with the third month in vitro, dispersed cells from both GLV-infected and control cultures were grafted into isologous adult W/Fu rats. Intraperitoneal, subcutaneous, intramuscular, and subrenal capsular inoculations were carried out; all results were negative.

When newborn rats were used as recipients for intraperitoneal cell transplantation, a typical viral-induced leukemia was obtained. It was considered as such because the main, and sometimes sole, localization was in the thymus, which resulted in a huge thymoma, and because the latency period of 75 to 90 days fell within the usual range of viral-induced leukemias. In addition, the inoculated cells were carrying GLV, which was obviously the inductive agent.

Conditioned adult W/Fu rats were then used as recipients after pretreatment with 450 r total body irradiation followed by three daily intramuscular injections (5 mg each) of cortisone acetate. The cultured cells were either grafted under the kidney capsule or injected intraperitoneally. Under these conditions, transplanted cells revealed their malignant potential and produced tumors, at the site of injection, that killed the recipient animals in 5 to 6 months. Before transplantation, the tumor-producing cultures had been growing in vitro for 4 months after the initial infection with GLV. Other cultures, after longer periods in vitro, were tested later and yielded similar results when they were grafted into the animals.

Most of the rats that received grafts of cultured cells under the kidney capsule displayed a local tumor. In addition, they manifested marked distention of the abdomen, as the animals that had been inoculated intraperitoneally also did; this was due to the accumulation of up to 30 ml of bloody ascites in the peritoneal cavity. The

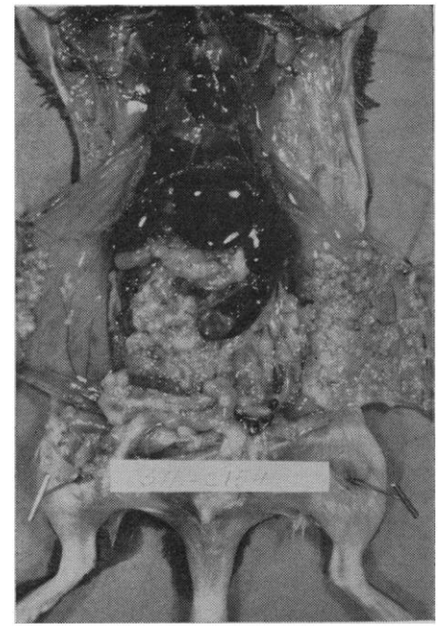


Fig. 3. W/Fu rat injected intraperitoneally with GLV-infected thymic culture (after 279 days in culture). Rat died after 246 days; bloody ascites and peritoneal tumors were present. The tumors cover parietal and visceral peritoneum. Thymus is not involved.

peritoneal surfaces, parietal and visceral, were covered with innumerable tumors of different sizes that were rounded, sessile, or pediculated; they filled the mesentery and covered the surfaces of most of the peritoneal organs (Fig. 3). Metastases were not found either above the diaphragmatic surface or deep in the parenchyma of the viscera. The thymus was involuted according to the age of the animal; the spleen and lymph nodes were not involved; and spreading seems to have taken place by local implantation.

Histologically, the pattern of the tumors resembles that of a reticulum-cell sarcoma (Fig. 4). The cells are large, epithelioid, polyhedral, or rounded; they are arranged in sheets and sometimes form pseudopapillary structures. The cytoplasm is variously acidophilic; the nucleus is large and nucleolated. Mitotic figures, more often atypical, are frequent.

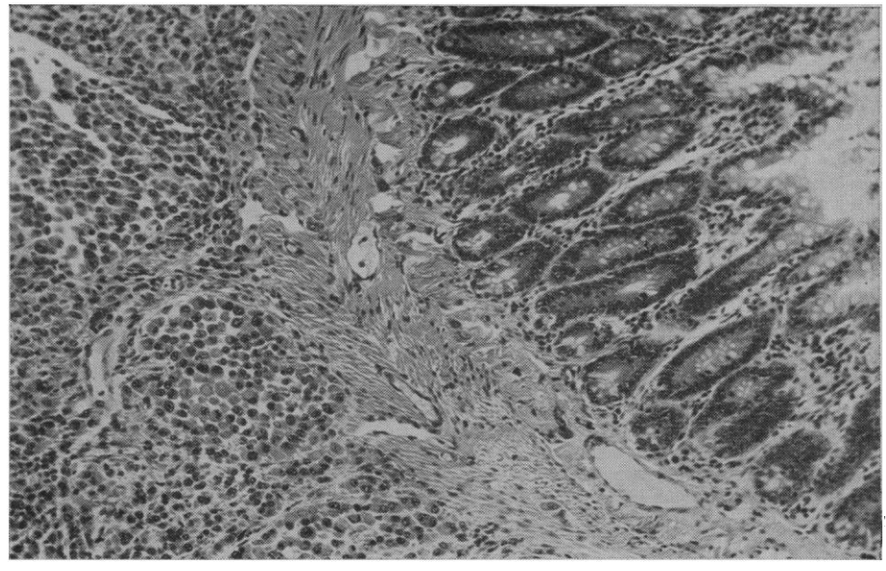


Fig. 4. Primary tumor (left) situated in the peritoneal serosa of the intestine. The glandular mucosa (right) and muscularis are not involved (× 200).

Small fragments of these primary tumors were grafted subcutaneously into adult nonconditioned W/Fu rats. All grafts were positive and each of the tumors, which became palpable in about 2 weeks, was subsequently serially transplantable. The histological appearance of the transplanted tumors was similar to that of the primary ones. A remarkable characteristic, persisting in all further grafted tumors, was the development of central cystic cavities containing up to 10 ml of clear yellowish fluid. Examination of tumors by electron microscopy in their third transplant generation demonstrated the presence of virus particles, similar to those previously identified in the cultures.

Since rats usually do not carry viruses, it is assumed that these virus particles originated by continuous replication of the GLV that was used initially to infect the thymic cultures. Thus, viral replication constantly took place in the thymic cells, both in vitro and in vivo, during a total period of about 20 months.

Concomitantly, the thymic cells acquired a malignant potential during their culture in vitro, which was revealed upon their transplantation in isologous recipients. Gross leukemia virus, which was present in large amounts in the cultures and has replicated constantly in the thymic cells, is considered the inducive agent of this cellular transformation.

The capacity to induce malignant cellular transformations in vitro was previously demonstrated for several oncogenic viruses: Rous sarcoma virus (*7*), polyoma virus (*8*), avian myeloblastosis virus (*9*), SV40 (*10*), and others. Manaker *et al.* (*11*) inoculated newborn mice with a 5-year-old culture that carried Moloney virus and local tumors developed, but the authors came to no conclusion as to the role of the virus in this induction of neoplasia. In a recent paper, Osato *et al.* (*12*) reported transformation of cells infected in vitro with Friend virus. However, in this case, the cultures lost their leukemogenic potential after the eighth subculturing, and the presence of virus could not be demonstrated either in cultures or in the fibrosarcomas that arose after grafting cultured cells in mice.

In contrast, in the experiments reported here, infectious virus particles were present continuously and were replicating in the thymic cells. Therefore, in this case, it seems that "spontaneous" cellular transformation (*13*) can be excluded and GLV can be considered the inducive agent.

Neoplastic transformation of thymic cells in vitro by GLV reveals a previously unknown potential of this virus and provides a system for further study. This system presents several advantageous characteristics for such studies: (i) Replication of infectious virus takes place constantly and accompanies the cellular transformation. (ii) The use of cells of rat origin avoids the interference of oncogenic viruses naturally carried by mice. (iii) Thymic cells are particularly apt to support replication of GLV in culture. After having been transformed in vitro, they induce, upon isotransplantation, tumoral growths that both grossly and histologically display particular characteristics.

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