An Electron Microscopic Study of Leukemia Induced
in Rats with Gross Virus*

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SUMMARY

The leukemia virus of Gross was adapted to W/Fu rats to which it became as highly pathogenic as to mice. The lymphomas produced in rats were invariably thymic with or without leukemia and involvement of other organs.

An electron microscopic study was made of abundance of virus in the blood and various organs. Virus was found most often (100 per cent) and in greatest abundance in the thymus. The earliest clearly recognizable change was "budding" on the plasma membrane associated with accumulation of virus between well preserved cells. At an advanced stage of the disease, cytopathic changes were evidenced by dissolution of the plasma membrane innumerable viral particles intermingling with mitochondria, and other cytoplasmic elements.

Intracellular viral particles were abundant in vesicles or canals and specific granules of megakaryocytes of the spleen and bone marrow, and few elsewhere in their cytoplasm. Conventional electron micrographs give no clue as to the primary site and mode of replication of viral nucleic acids.

The viral particles seen resembled those of other leukemias. Some possessed tail-like structures as first described by Dalton for the Moloney virus (3, 4).

By fractionation of the blood plasma a nearly pure preparation of viral particles was obtained. This procedure was adapted to the thymus by dissociation of thymic cells by treatment with trypsin (instead of homogenization), followed by fractional centrifugation.

The abundance of virus and the neoplastic transformation of lymphocytes in the thymus may be related to the presence of a specific factor in this organ.

It has been well established that the induction of lymphoma in mice by virus is dependent on a thymic factor (cf. 12) yet to be identified. The many excellent electron microscopic studies on leukemias of murine origin have been surveyed by others (3, 5–8). There are, however, no precise data on the relative concentration of virus in the blood and various organs. Especially wanting are data on changes in the thymus in relation to the presence of the virus. To fill this gap a series of experiments was undertaken. Those to be reported here are concerned with the concentration and location of the virus as disclosed by electron micrography, in the blood, thymus, and several other organs in lymphomatous W/Fu rats. Thymic lymphoma was induced by Gross virus adapted to rats ca. 3 years ago.

MATERIALS AND METHODS

Animals.—Newborn rats of W/Fu strain (15) were obtained by random breeding in our laboratory and given injections when 1–3 days old. Nursing mothers received bread and milk daily. The virus-injected rats were weaned and males and females separated at about 4 weeks of age. The rats were maintained on Purina chow and water ad libitum. Intercurrent infections were treated with Terramycin (animal formula, Pfizer) in the drinking water and on a few occasions by a single injection of Bicillin (Wyeth).

Virus.—Passage A virus of Gross (13) has been maintained in our laboratory since 1957 through successive passages, first in mice and later in rats.
The preparation and storage of the filtrate containing the virus have been described (17).

Adaptation of the passage A virus to W/Fu rats.
—In the first mouse-to-rat passage, eleven of nineteen 1- to 92-day-old W/Fu rats given injections intraperitoneally with 0.2-0.3 ml. of the filtrate developed thymic lymphoma with or without generalized lymphoma after an average latency period of 143 days. In the first rat-to-rat passage, leukemia developed in sixteen of seventeen rats (94 per cent) given injections at an average latency period of 86 days. In the next passage 48 of 51 rats (94 per cent) and in the subsequent rat passage all infected animals developed localized or generalized thymic lymphoma after average latency periods of 85-79 days. The potency of the rat passage virus increased further through serial passages in rats and became as potent in rats as in mice. The shortest latency period was 50 days.

Inspection of infected rats.—After being weaned the rats were inspected weekly, with development of anemia, emaciation, or dyspnea watched for particularly. Blood counts and blood smears were made when leukemia was suspected.

Advanced thymic lymphoma was readily recognized from the bulging chest. Rats diagnosed as having thymic lymphomas usually died within 1-2 weeks. The most reliable diagnostic procedure is a careful hematological examination. Large (leukemic) lymphocytes, rarely seen in normal blood, are present in the majority of lymphomatous rats but usually in small numbers only. Soon after a definitive diagnosis of lymphoma was made the rats were sacrificed.

X-radiation of leukemic rats.—To study the effect of x-rays on the distribution and quantity of the virus in leukemic rats, several matched pairs were separated. One rat was given 350-500 r total-body x-radiation, and the pair was sham-irradiated or left without treatment. The peripheral blood of both was examined periodically. The pair was sacrificed 5-8 days after radiation when one became moribund. The factors of radiation were 300 kv., 15 ma., 0.5 mm. Cu + 1.0 mm. Al filtration; target distance, 50 cm. HVL, 1.1 mm. Cu and air dose rate, 48.1 r/min. All rats of this series were treated with Terramycin in the drinking water from the day of irradiation until sacrifice.

Collection of material and preparation for electron microscopy.—The technic of obtaining virus-containing pellets from the plasma dated to experience of one of us (14) that avian leukemia virus is present in the plasma in high concentration and can be sedimented by high-speed centrifugation. This technic was later perfected by Beard and his associates, and the presence of virus in their pellets was demonstrated in electron micrographs by Bernhard et al. (1). This was soon confirmed by us, and the procedure was also recommended (10, 11) and tried for search of virus in plasma of leukemia patients. No difficulty was encountered to obtain almost pure preparation of virus from the plasma of viral leukemic mice and rats. A similar technic was worked out by Moloney (7).

Rats to be sacrificed were heparinized by intravenous or intraperitoneal injection, anesthetized with ether, exsanguinated by cutting an external jugular vein, and then perfused with physiological saline injected into a femoral vein. About 30 ml. of blood with perfusate per animal were centrifuged at 3000 r.p.m. for 20 minutes in a refrigerated centrifuge (Model PR-2, Rotor 253, International). The supernatant fluid was decanted and recentrifuged at 7000 r.p.m. for 30 minutes (Rotor type SS, Servall) in a cold room (4° C.). The final centrifugation was done at 40,000-45,000 r.p.m. for 1 hour (Model L, Rotor 50, Spinco). The supernatant was discarded, and the pellet obtained was processed as follows.

Preparation of virus-pellet from thymoma.—Since most virus particles were found in intercellular spaces of thymoma, a method was devised to obtain a virus-pellet from the thymomas. Instead of homogenization, thymic cells were dissociated by gentle treatment with trypsin (as done for tissue cultures), the cells were centrifuged off, and the virus was collected by differential high-speed centrifugation as described above.

Fixation and embedding for electron microscopy.—The plasma-pellet and pieces of thymus, spleen, and bone marrow of all cases, and the lymph nodes and kidney in a few cases, were processed as follows:

From each organ, 20- to 30-minute pieces were fixed in a bottle containing 4 ml. of 1 per cent osmium tetroxide solution in veronal-acetate buffer (pH 7.6) to which 0.196 gm. of sucrose was added (2). The bottles were placed in an ice-cold water bath for 1½ hours. The fixed tissues were dehydrated with a graded series of either acetone or ethyl alcohol, beginning with a 25 per cent concentration. About ten pieces of each tissue were embedded in Araldite in No. 4 gelatine capsules.

The pellets from either plasma or thymus were fixed and dehydrated in the same manner as the tissues. To facilitate processing, the part of the cellulose tube holding the pellet was cut off, and the pellet in it was fixed. After dehydration the pellet was carefully removed from the cellulose tube which had become softened through dehy-
over-all larger size of leukemic cells. Fine nuclear chromatin is concentrated in both peripheral and central areas of the nucleus. The cytoplasm contains scattered endoplasmic reticulum, evenly distributed cytoplasmic ribonucleoprotein granules, and few round or oval mitochondria. When the cells are intact the virus particles are located only in intercellular spaces, sometimes in small (Fig. 3) and more often in large numbers (Fig. 4). It seems to us that there is a relation between the abundance of virus and the state of leukemic cells. When virus particles become more abundant, the leukemic cells lose their cytoplasmic membranes partially or completely, and their cytoplasmic constituents are intermingled with large clusters of virus. In extreme cases pyknotic nuclei and cytoplasmic components such as mitochondria are embedded in large clusters of virus (Fig. 5). This can be regarded as a cytopathic change secondary to heavy “infestation” of the organ with virus.

In ultrathin sections of thymus in irradiated rats, large cells, either macrophages or of epithelial-reticulum type, are often observed among leukemic cells. They have large cytoplasm in which much endoplasmic reticulum, oval mitochondria, electron-dense areas, and virus-containing vacuoles can be observed (Fig. 6). Transitional forms of these structures suggest either formation of virus in mitochondria or a degenerative change of virus trapped in vacuoles. The rarity of similar changes in nonirradiated rats suggests that these changes (Fig. 6) may be related to radiation.

In ultrathin sections of spleen and bone marrow the extracellular virus particles identical with those observed in thymuses can be observed among infiltrating leukemic cells, singly or in a small group of three or four viruses. Even in massively infiltrated spleens such a large cluster of virus particles as in thymus was never observed.

As first reported by de Harven and Friend (6), megakaryocytes of spleen and bone marrow contain considerable numbers of virus particles, most of which are characterized with the doughnut-like structure (Figs. 7, 8). The fine structure of the area of megakaryocytes containing virus is somewhat different from that of normal megakaryocytes (Fig. 10a). The most conspicuous difference is the scarcity and appearance of specific granules of megakaryocytes containing many viruses. The virus particles are usually located in canals or vesicles and specific granules, and some are apparently free in cytoplasm (Figs. 7, 8). The appearance of virus-containing megakaryocytes was more fully described by Dalton et al. (5) and Dmochowski et al. (8).

**Results**

**Viral lymphomas of the rat.**—Since this virus was adapted to rats approximately 250 lymphomas induced with it were studied. Of these, 22 were electron-micrographed in the present series with seven normal rats. The thymus was invariably involved (Fig. 2a), whereas in other organs the infiltrations varied greatly in intensity, and in ca. 20 per cent of the cases none was observed.

The neoplastic cell resembled normal lymphoblasts (Figs. 2b, 2c). Although myeloid leukemia and reticulum-cell sarcoma are known to occur in this strain, none was seen in this series. (Experiments are in progress to test their inducibility by thymectomy of virus-infected rats.) The present series did not suggest that this virus is capable of producing any neoplasm in the rat other than lymphoma of thymic origin.

**Electron microscopy of thymus, spleen, and bone marrow.**—In ultrathin sections the leukemic cell population of the thymus (Fig. 3) resembles the normal cell population (Fig. 1), except for the over-all larger size of leukemic cells. Fine nuclear chromatin is concentrated in both peripheral and
in which virus particles were found invariably. Tables 1, 2, and 3 show their frequency and abundance in various organs as observed by electron microscopy. Sections of thymomas of all 22 leukemic rats showed intercellular virus particles in varying concentrations (Figs. 3-5).

Contrary to expectation (19), the thymomas of leukemic rats, treated with 850-500 r, seemed to contain fewer virus particles than do their nonirradiated pairs (Tables 2, 3). This discrepancy remains to be explained.

In the spleen and bone marrow extracellular virus was seen in small numbers and in only six of fourteen spleens and in six of fourteen bone marrows examined. Intracellular virus was seen in megakaryocytes in four of fourteen spleens and in one of fourteen bone marrows examined (Tables 1, 3). The virus particles seen in spleen and bone marrow of the irradiated leukemic rats were all extracellular and few. Only two spleens and four bone marrows of the eight cases examined had virus particles (Tables 2, 3).

Four of eleven plasma pellets from nonirradiated rats (Tables 1, 3) contained virus in various concentrations. In one case the plasma pellet failed to show the virus, but the platelet fraction contained much virus intermingled with disintegrated platelets (Fig. 10b). The possibility that virus adheres to platelets as earlier described by Dalton et al. (5), notably when they are disintegrating, is being currently studied.

### TABLE 1

ABUNDANCE OF VIRUS AS SEEN IN ELECTRON MICROGRAPHS; HEMATOLOGICAL AND OTHER DATA ON RATS WITH VIRAL LYMPHOMA

<table>
<thead>
<tr>
<th>No.</th>
<th>LATENCY (DATE)</th>
<th>Plasma pellet</th>
<th>Thymus E</th>
<th>Thymus L</th>
<th>Spleen E</th>
<th>Spleen L</th>
<th>Bone marrow E</th>
<th>Bone marrow L</th>
<th>Lymph node E</th>
<th>Lymph node L</th>
<th>Liver L</th>
<th>ANOMALOUS LYMPHOCYTES (PER CENT)*</th>
<th>EXTRANEOUS CELLS (PER CENT)</th>
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<td>±</td>
<td>±</td>
<td>±</td>
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<td>1 (0-3)</td>
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<tr>
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<td>-</td>
<td>±</td>
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<td>±</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+++</td>
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<td>+</td>
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<td>1 (0-3)</td>
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<tr>
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<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>11.0 (0-460)</td>
<td>1 (0-3)</td>
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</tbody>
</table>

* This column includes all lymphoid cells with nucleoli and atypical, presumably leukemic, lymphocytes.
† Killed by mistake; thymic lymphoma was confined in one lobe.
‡ The platelet fraction contained virus (+++).

Abbreviations: E = Abundance of virus as seen in electron micrographs. - = none; ± = few; ++ = many; +++ = very many. L = Degree of lymphomatous infiltration as indicated by light microscopy. - = none; + = slight; ++ = moderate; +++ = marked. m = Virus particles in megakaryocyte. i = Virus particles in intercellular space.

Thus, virus was found more often (71 per cent) in the plasma pellets in irradiated than in nonirradiated rats (36 per cent) (Tables 2, 3).

The findings suggest the possibility of enhancing viremia by radiation. This may be caused by suppression of antibody production and (or) release of virus from lymphomatous infiltrations destroyed by irradiation. Clarification of this problem requires further work.

It is noteworthy that a vast amount of virus remained in tissues following exsanguination-perfusion and could be collected by the trypsinization procedure described (Fig. 11b).
### Table 2

**Effect of Radiation on Abundance of Virus as Seen in Electron Micrographs and on Hematologic and Other Findings in Rat with Viral Lymphoma**

<table>
<thead>
<tr>
<th>No. Rat</th>
<th>Pair in Table 1</th>
<th>Lateness (Days)</th>
<th>Sacrifice (Days)*</th>
<th>Plasma pellet</th>
<th>Thymus E L</th>
<th>Spleen E L</th>
<th>Bone marrow E L</th>
<th>Lymph node E L</th>
<th>Liver L</th>
<th>WBC (ct. mm.) ( \times 10^3 )</th>
<th>Abnormal lymphoid cells (per cent)?</th>
<th>Erythroblasts (ct. mm.)</th>
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<td>19.7</td>
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<td>±</td>
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<td>i+</td>
<td></td>
<td>20.2</td>
<td>0.4</td>
<td>7,700</td>
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**Controls:**

| 23      | -              | 8              | -                 | -            | -         | -         | -              | -              | -      | 13.1                       | 3.5                      | 0,4                       | <10                      |
| 24      | -              | 8              | -                 | -            | -         | -         | -              | -              | -      | 23.5                       | 4.0                      | 2,4                       | <10                      |

*After irradiation.

† See footnote to Table 1.

For abbreviations and signs see footnote to Table 1.
Morphology of virus particles.—There are several reports on the micromorphology of the Gross leukemia virus (cf. 7, 13). In general our observations duplicate those of others. The virus particles observed in our ultrathin sections of various organs could be divided into four types:

a) Moderately electron-dense particles measuring 80–100 mμ in diameter, with distinct inner and outer membranes and no nucleoid.

b) Particles with an electron-dense nucleoid centrally or excentrically placed, and obscure inner membrane, also measuring 80–100 mμ in diameter.

c) Particles usually without a distinct nucleoid but with similar tail-like structures (Fig. 9) as described earlier by Dalton (3, 4) in Moloney virus, without nucleoid and an electron-dense zone between outer and inner membranes, thus forming doughnut-like structures. The overall diameter of these particles ranges from 95 to 105 mμ; their central zone measures approximately 50 mμ. They were usually found within small vesicles or canals and specific granules in the cytoplasm. Some of them appear budding into vesicles from smooth-surfaced endoplasmic reticulum.

Hematologic findings.—These are surveyed in Tables 1 and 2. A remarkable observation was the presence, in the blood, of large numbers of small lymphocytes possessing nucleoli. The latter, we found, was a better diagnostic criterion of lymphoma than the presence of elevated numbers of large lymphocytes. Lymphocytes with nucleoli

<table>
<thead>
<tr>
<th>TABLE 3</th>
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<td>SUMMARY OF TABLES 1 AND 2. INCIDENCE AND ABUNDANCE OF VIRUS IN LEUKEMIC RATS</td>
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<table>
<thead>
<tr>
<th>Rats</th>
<th>Plasma</th>
<th>Thymus</th>
<th>Spleen</th>
<th>Bone Marrow</th>
<th>Lymph node</th>
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<td>Nonirradiated</td>
<td>Incidence</td>
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<td>14/14</td>
<td>14/6</td>
<td>14/6</td>
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<tr>
<td></td>
<td>Abundance</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Irradiated</td>
<td>Incidence</td>
<td>7/5</td>
<td>8/8</td>
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<td>+</td>
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</tbody>
</table>

* Number examined over number positive.

For abbreviations and signs see footnotes to Table 1.

protruding from the angular or hexagonal “head” about 100–200 mμ in diameter. The tail is ca. 100 mμ in length and 50 mμ in width. The outer coat of the tail is continuous with the outer membrane of the head. The outer membrane of these three types of virus particles consists of two layers. It appears to be essentially identical with the cytoplasmic membrane in its fine structure (Fig. 9). These three types of virus particles were observed mainly extracellularly in lymphomatous thymus, lymph node, spleen, and bone marrow, either singly (Fig. 3) or in a large cluster (Figs. 4, 5) and in plasma pellets (Fig. 11a).

d) The fourth type of virus was seen abundantly in the megakaryocytes of spleen and bone marrow (Figs. 7, 8). These particles have distinct inner and outer membranes; they are oval or spherical, and have an electron-lucent center

COMMENTS

The thymus plays a determining role in lymphomogenesis by virus, chemical carcinogens, and radiations (cf. 12). The present experiments are
Fig. 2a.—Localized thymic lymphoma of a rat induced by virus.
Fig. 2b.—Same. X750.
Fig. 2c.—Imprint of the thymic lymphoma of a rat 8 days following irradiation with 330 r. X1400. (Cf. Fig. 6a.)
Fig. 2d.—Megakaryocytes of the spleen of a lymphomatous rat. X400. (Cf. Fig. 7.)
Fig. 3.—Electron micrograph of the thymus of a rat with thymic and generalized lymphoma. Note the excellent preservation of the leukemic lymphocytes and the location of a few viral particles scattered in intercellular spaces. One arrow points to “budding” of the plasma membrane. ×24,000.
FIG. 4a.—Large numbers of viral particles between well preserved lymphoblasts of a thymic lymphoma. $\times 15,000$.

FIG. 4b.—Phase-contrast photograph taken from the same block. $\times 900$. 
Fig. 5.—Electron micrograph of thymic lymphoma of a rat that also had a generalized lymphoma with mild leukemic blood picture. ×19,200. The plasma membrane of most cells is gone, the viral particles extend as far as the nuclear membrane, and mitochondria (m) lie free between viral particles. This picture was taken from an area where light microscopy indicated degeneration. n = nucleus.
FIG. 6a.—Viral particles (V) filling vacuoles in the cytoplasm of a large cell of the thymus. This rat was given 350 r 8 days before sacrifice and had generalized lymphoma. The character of this cell is uncertain. It is either a macrophage as shown in Figure 2c or an epithelial- reticulum cell. \( \times 38,000 \).  

\( \pi \) = nucleus.

FIG. 6b.—Phase contrast photomicrograph from same block as 6a cut about ten times as thick. \( \times 880 \).
Fig. 7.—Numerous viral particles in megakaryocyte of the spleen of the rat that had thymic and generalized lymphoma, ×38,000.
FIG. 8.—Megakaryocyte of the bone marrow of a rat that had thymic and generalized lymphoma. The megakaryocyte is full of viral particles, but only a few are seen in intercellular spaces in the bone marrow. $\times 53,000$. Arrows point to “bud-ding.” $n =$ nucleus, $c =$ probably centriol.
Fig. 9a.—Higher magnification of Figure 4a showing the structure of the viral particle. One arrow points to the plasma membrane and others to tail-like structures of the virus. X150,000. C = cytoplasm.

Fig. 9b.—"Budding" on the plasma membrane. X50,000.

Fig. 9c.—Shows the varied appearance of the virus. X74,000.
Fig. 10a.—A megakaryocyte of a normal bone marrow of a rat of the W/Fu strain showing the platelet mosaic of the cytoplasm. X12,000.

Fig. 10b.—Platelet fraction of a leukemic rat obtained by differential centrifugation. The white cell count of this animal was 114,400. The blood smear showed larger numbers of pyknotic cells. In the electron micrographs the thymus was loaded with viral particles. The plasma pellet from this case contained none indicating adherence of the viral particles to disintegrating platelets. X15,000.
Fig. 11a.—The plasma pellet of a leukemic rat obtained by differential centrifugation. Note the unusual purity of the viral preparation. This rat had only a localized thymic lymphoma and received 500 r 6 days before sacrifice. × 25,500.

Fig. 11b.—A pellet of almost pure virus preparation obtained from thymuses of leukemic rats by the procedure described in the text, showing the absence of cell debris which is usually abundant in preparations from organ homogenates. ×24,100.
part of a systemic study for attempting to elucidate this relationship. It is now well known that thymectomy prevents lymphoma induction and grafts of normal thymus correct this deficiency (cf. 12, 13).

The present studies suggest that the leukemic thymus contains more virus than does any other organ. The presence of leukemia virus in numerous organs was reported by others (5, 8, cf. also 7, 13), but it is not evident that they have made a comparative study of various organs. Further, host and virus were not the same in their study. Experiments of Krischke (16) suggest that the concentration of leukemia virus increases gradually from the day of infection, reaching the maximum at time of onset of leukemia. It is now well known that virus is present in megakaryocytes which are not known to undergo leukemic transformation. We have also seen them in megakaryocytes of both bone marrow and spleen. The electron micrographs show pictures of budding in vesicles and canals of these cells; however, similar budding from the plasma membrane of other cells has also been noted. In the present study this was conspicuous in the thymus. Earlier, Edwards (9) noted them in parotid tumors of nonleukemic Ak hybrids which are known to carry the leukemia virus. The sites of replication of viral nucleic acids are unknown, and the "budding" phenomenon is usually attributed to acquisition of a protective coat by the virus.

It is remarkable that the bulk of the virus is intercellular. The question arises whether virus multiplies in this location or is merely trapped there. The "budding" phenomenon on cell surfaces is interpreted as "coat formation." The site and mode of replication of viral nucleic acids are still a mystery.

The abundance of virus in the thymus may be linked to the thymic factor which brings about lymphomatous transformation. Other sites of lymphomatous infiltration do not show virus in like abundance.

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