Electron Microscopic Study of Lymphatic Tissues of Mice Inoculated with a Leukemogenic Extract from Radiation-induced Tumors

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SUMMARY

Macroscopic, histological, and ultrastructural changes in lymphatic tissues, particularly that of the spleen, were studied in C57BL mice inoculated i.p. with a cell-free extract (Passage D from Dr. Duplan) of lymphatic tissues from mice in which leukemia was originally radiation induced.

Splenomegaly was observed macroscopically, followed by hypertrophy of lymph nodes. But no change of the thymus was apparent throughout the course of this infection. The histological appearance of the spleen showed hyperplasia of the germinal centers within the lymphatic nodules. The lymphoid cells were replaced progressively by proliferation of the reticular cells and, to a lesser extent, by cells of the plasmocytic line.

Typical type C particles were found in the splenic white pulp. During the early stages of this disease, the particles were concentrated in the extracellular spaces within the germinal centers. The infected cells were predominantly immunoblasts, plasmoblasts, plasmocytes, and to a lesser extent lymphoblasts, lymphocytes, and reticular cells. Viral particles could not be detected within megakaryocytes or erythrocytic precursor cells. A few intracisternal type A particles were observed in both plasmocytes and reticular cells. The characteristics of this leukemia are discussed in relation to those described by Kaplan and Rauscher.

INTRODUCTION

A very low incidence (+6%) of spontaneous thymic lymphomas was observed in C57BL mice by Kaplan and Brown (12). However, after fractionated γ-radiation the incidence of thymic lymphomas in C57BL mice is increased to more than 90%.

Kaplan has shown that the thymus is the target organ responsible for leukemogenesis (9) and that thymectomy strongly decreases the incidence of mouse radioleukemia (10). It has also been shown that cell-free extracts of leukemogenic lymphoid tissues can transmit the disease (8, 13). A virus called RadLV is the agent responsible for the induction (11) and its virulence can be increased by successive host-to-host passages (5).

Recently, Dr. J. F. Duplan (Fondation Bergonié, France) (personal communication) was able to induce a reticular cell sarcoma of the spleen and lymph nodes in C57BL mice that had been inoculated with an extract (called Passage D) prepared from lymphatic tissues of leukemic mice. These donor mice became leukemic as a result of γ-radiation, following the classic scheme of Kaplan. The purpose of the present study was to determine, at a macroscopic, histological, and ultrastructural level, the pathological changes within the lymphoid system of C57BL mice during the early stages of Passage D-induced leukemia.

MATERIALS AND METHODS

Animals. We used 4-week-old male C57BL mice. The RadLV agent (Passage D) was easily adapted to this strain.

Two groups of 200 mice each were used to study the survival time of leukemic and control mice, respectively.

Further groups of mice were used to investigate the morphology of various tissues and to observe weight changes of the spleen over a 5- to 85-day period. Some mice were sacrificed after more than 85 days for additional morphological studies.

Viral Extract. The highly pathogenic cell-free extract, Passage D, which we used in this study, was obtained from Dr. Duplan. It is an activated subline of a virus RadLV originally isolated from the lymphatic tissues of a C57BL mouse in which leukemia was induced by 4 doses of 175 R given at weekly intervals. In an attempt to increase the virulence of the original RadLV, serial host-to-host passages were made by Duplan. The 1st and 2nd passages of this agent were made by the inoculation of a cell-free extract; the 3rd and 4th were made by the s.c. transplantation of a fragment of leukemic tissue. A cell-free extract prepared from the leukemic mice of the 4th passage was injected via different routes into 4 groups of mice giving rise to sublines A, B, C, and D. The most virulent of these sub-
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lines, later designated as the Passage D, resulted from the i.p. inoculation of the extract into thymectomized C57BL mice (15).

In our studies the viral extract was prepared from spleen and lymph nodes of leukemic mice in the terminal stages of the disease. Tissue fragments were weighed, minced, and ground in a porcelain mortar and precooled at -18°. This cell preparation was suspended in phosphate-buffered saline at 4° (1/5, w/v) and centrifuged for 15 min at 5,000 × g in a Sorvall refrigerated centrifuge. The supernatant was centrifuged for 35 min at 16,000 × g. This cell-free supernatant constituted the extract. It was immediately injected into mice or stored in liquid nitrogen. The mice were inoculated i.p. with 0.5 ml of this extract. A cell-free extract of lymphoid tissue was also prepared from nontreated mice and injected into a group of 30 mice.

Light Microscopy. Histological investigations were made of the spleen, lymph nodes, thymus, liver, kidneys, and lungs from both leukemic and control mice. Most tissues were fixed in Bouin-Hollande solution. Hematoxylin-eosin-saffran and methyl green-pyronine were used for routine staining. Sections were also stained with the silver impregnation method (20) to visualize the reticulin fibers.

Electron Microscopy. In both leukemic and control mice spleen, lymph nodes, thymus, and bone marrow were sampled at several intervals between 2 hr and 29 days after the extract inoculation. Organ fragments were fixed in buffered osmium tetroxide solution (16), dehydrated, and embedded in Epon 812 (14). Sections were cut with an LKB III Ultrotome microtome equipped with a diamond knife. Thick sections, for orientation by light microscopy, were stained with toluidine blue solution. Thin sections of about 500 to 700 A were mounted on uncoated copper grids and stained with aqueous solutions of uranyl acetate and lead citrate (17). They were examined in a Philips EM-300 electron microscope at 60 kV.

RESULTS

Development of the Disease

The incidence of spontaneous leukemia in C57BL mice is 5%. At the 1st passage approximately 20% of the mice given injections of the extract prepared by Dr. Duplan were leukemic. After several host-to-host passages and the selection of those mice that first exhibited macroscopic signs of leukemia, the pathogenic potency of the virus had increased and a 100% incidence was obtained. Leukemia could be induced regularly after a relatively short latency period (1 month). Slight splenomegaly was apparent 25 days after injection. This was concurrent with or followed by the enlargement of peripheral lymph nodes, particularly those of the axillary and inguinal pits. An increase in spleen weight was apparent on Day 10 post inoculation and reached a maximum on Day 50. The average maximum spleen weight of leukemic mice reached 900 mg, whereas those of the control mice was only 100 mg (Chart 1).

At autopsy, all the leukemic mice showed splenomegaly and hypertrophy of all the lymph nodes (inguinal, axillary, mediastinal, mesenteric, submaxillary, etc.). The magnitude of these symptoms was a function of the development of the disease.

On the other hand, throughout the development of the infection, the thymus of a leukemic mouse remained similar in macroscopic appearance to that of a control mouse.

After a 6-month period of observation, the mice treated with a cell-free extract prepared from control mice showed none of the symptoms associated with leukemia induced by Passage D extract.

Histology

Histological changes in the spleen of mice treated with the viral extract consisted mainly of a hyperplasia of the germinal centers of the lymphatic nodules. The lymphoid cells were progressively replaced by reticular cells proliferating in an abnormal manner and to a lesser extent by cells of the plasmocytic line.

On the 5th day, only small changes could be observed in the spleen nodules; a few immunoblast cells were present and the number of reticular cells was slightly increased.

On the 10th day, the germinal centers were somewhat enlarged and the number of reticular and immunoblast cells was further enhanced (Figs. 1 and 2).

On the 15th day, proliferation of reticular cells and cells of the plasmocytic line resulted in some disorganization.
tion of the splenic nodules (Figs. 3 and 4). The small lymphocytes of the cortical area were reduced in number.

After 30 days, reticular cells in the germinal centers had proliferated to such an extent that they had displaced the red pulp of the spleen toward the periphery (Fig. 5). Reticular cells also proliferated within the red pulp.

The staining of reticulin fibers revealed a consistent pattern; they occur as a network at the center and the periphery of the disorganized splenic nodules instead of being localized at the nodule periphery as in normal spleen (Figs. 6 and 7).

The same histological changes appeared in the lymph nodes; the germinal centers became hyperplasic and the normal structure of the nodes could no longer be discerned from the 30th day on.

The thymus behaved differently and displayed an infiltration of neoplastic cells only at a later time. Thus its structure is not much modified. Even on the 60th day, the cortical thymocytes, although less numerous, were still present.

In the kidneys and liver, the infiltration of neoplastic cells is observed from the 30th day but it is limited to the perivascular area.

Ultrastructural Studies

Ultrastructure of the Virus

The virus is a typical type C particle (1, 3), with a central core of electron-dense material, about 50 to 60 nm in diameter, containing the viral RNA. This is surrounded by an envelope that exhibits a typical unit membrane structure (Figs. 8 to 11). The overall diameter is about 90 to 100 nm (Fig. 11). This is the so-called extracytoplasmic “mature form” type C particle (19). The other form appears as a ring-shaped particle with an electron-lucent central core and 3 distinct concentric shells (Fig. 10); this is the immature form, the term being used without any implication as to its infectivity. The type C particles are produced by budding along the cell membranes of different kinds of cells (Figs. 8 and 9). The budding is not the sole property of the plasma membrane; it occurs also along smooth membrane limiting cytoplasmic vacuoles (Fig. 12). In lymphoid tissue of injected animals the mature form appears as the most frequent particle.

Another type of particle, the “intracisternal type A particle” (19), has been detected mainly in plasmocytes and reticular cells. It consists of 2 concentric shells of equal thickness with an electron-lucent center of 30 to 40 nm. It has an overall diameter of only 70 to 80 nm. These viruses have an intracellular localization; they bud from the membranes of the rough and smooth endoplasmic reticulum and are released into the reticulum cisternae of the cells (Figs. 13 to 16). These type A particles are seldom seen.

Virus Localization

Spleen. Type C particles were detected in the spleen of infected mice at each time interval studied. Very few were found in the red pulp but many were present in the white pulp. From the 3rd day on the virions were found to be mainly concentrated in the germinal centers of the lymphatic nodules. Most of them were found in extracellular spaces, surrounded by the plasma membrane infoldings of the reticular cells, which have been designated the Antigen-retaining reticulum cells (6) (Fig. 17).

In the samples taken between 6 and 12 days after injection, most of the viral particles were still located in the extracellular spaces of the germinal centers, but in fact, many cells showed virus budding along their plasma membrane and were already infected. The infected cells were mainly those of the plasmocytic and lymphocytic lines and to a lesser extent reticular cells (Figs. 18 to 22).

Around the 14th day, immunoblasts, plasmoblasts, and plasmocytes are the most heavily infected cells. From Day 22 on, the number of extracellular viruses as well as the number of infected cells had increased. Of 2500 cell sections screened for budding particles, approximately 100 cell sections were infected after 29 days. A total of 225 type C particles were seen within this population; the majority were extracellular and mature and only 2% were situated intracellularly within cytoplasmic vacuoles. The cell population of the spleen underwent definite changes from Day 30 on. The new population of the lymphatic nodules consisted mainly of the reticular cells and cells of the plasmocytic line. Small lymphocytes were rarely seen (Fig. 23).

Thymus, Lymph Nodes, and Bone Marrow. Many type C particles were present in the lymph nodes of the leukemic animals. As in the spleen, the virus was budding from the membranes of the plasmocytic and lymphocytic line (Figs. 24 and 25).

The type C particles were scarce in the thymus. The virus could not be detected in the bone marrow, even long after extract inoculation. Budding from the membranes of megakaryocytes and erythroblasts was not observed. As described in the literature (2, 4) few type C particles were seen in tissues of nontreated C57BL mice.

DISCUSSION

Inoculation of C57BL mice with Passage D extract of Dr. Duplan causes a reticulosarcoma of the spleen and lymph nodes. The reticulosarcoma originates in the germinal centers of the lymphoid nodules.

Ultrastructural examination of the germinal centers of the spleen reveals the presence of type C particles from the 1st days after viral infection. These particles are extracellular and concentrated along the infoldings of the plasma membrane of the reticular cells (18). Hanna et al. (6) had reported a similar observation for infection with the Rauscher leukemia virus. He suggested that the accumulation of leukemia viruses in the germinal centers might result in a direct antigenic stimulation of immuno-competent cells.

Hanna et al. (6) also published electron micrographs showing virus buddings from the immunoblasts plasma...
membrane. Since immunoblasts migrate from the germinal centers to the splenic red pulp, he proposed that the infected immunoblasts could be a source of viral material for the erythroblasts and megakaryocytes that became infected some days later. We also observed infected immunoblast cells (Fig. 22). However, the screening of the splenic red pulp of C57BL mice, inoculated with Passage D extract, failed to detect any infection of megakaryocytes and of the erythroblasts at any time interval studied. No erythroid hyperplasia could be demonstrated histologically.

Some other cells of the white line were observed to be able to replicate the virus. Among them are the small lymphocytes and the plasmaocytes which are generally considered not to be a virus multiplication site (including nononcogenic viruses) (7). Immunoblasts, plasma blasts, and plasmocytes appear to be the cells which produce the largest number of type C particles and might thus constitute the target cells for virus multiplication. The reticular cells scarcely show budding along their plasma membrane and might thus be considered to be the target cells for viral transformation.

Type C particles were easily observed in the spleen and lymph nodes of mice inoculated with the Passage D extract, whereas the screening of the thymus scarcely revealed the presence of virions. This observation together with the results of the histological examination confirmed that the thymus is not the target organ in the case of this leukemia, although the disease was originally radioinduced.

The differences existing between the leukemia induced by Kaplan’s RadLV and that elicited by the Passage D extract do not consist solely in target organs and target cells. Other biological parameters, e.g., the latency period, the rate of mortality, the dilution of the virulence of the extract, and the maximum age of susceptibility of the mouse, allow a clear distinction to be made between both types of leukemias.

The age of the mouse at the time of inoculation plays an important role in the induction of the disease. Kaplan (11) induced the highest percentage of leukemias (79%) by treating newborn mice. The treatment of 1-month-old mice was little effective. In contrast we were able to obtain 100% leukemia by treating 1-month-old mice with the Passage D extract.

The latency period preceding the clinical symptoms of the disease is about 100 days in mice infected with Kaplan’s RadLV, whereas it is only about 30 days for mice treated with Passage D extract. The acellular extract of Kaplan’s RadLV loses its leukemogenic activity after a 5- to 10fold dilution (11), while a 10-3 dilution of the Passage D extract is still active (P. Ricciardi-Castagnoli, J. M. Jadin, and J. R. Maisin, unpublished data).

The differences between both extracts (Kaplan’s RadLV and Passage D of Duplan) might be explained on the basis of their origin. Passage D was obtained by inoculating thymectomized adult C57BL mice with an acellular extract, prepared from mice bearing extrathyroid lymphoid tumors. Originally the leukemia was radiation induced (J. F. Duplan, personal communication).

Kaplan obtained his RadLV extract by inoculating newborn C57BL mice with a cell-free extract prepared from radiation-induced thymic lymphomas. For the successive passages the extract was also prepared from neoplastic thymic tissues (13).

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Fig. 1. Control mouse spleen lymphatic nodule with a germinal center. H & E, × 110.

Fig. 2. Spleen lymphatic nodule 10 days after inoculation with Passage D extract. Hyperplasia of the germinal center is already apparent. H & E, × 100.

Fig. 3. Spleen lymphatic nodule 15 days after inoculation with Passage D extract. Note the disorganization of the nodule and the depletion from small lymphocytes. H & E, × 70.

Fig. 4. Higher magnification of Fig. 3. Note numerous mitosis and immunoblast cells (arrow). H & E, × 320.

Fig. 5. Spleen section of a mouse, sacrificed 2 months after inoculation with Passage D extract. Note the complete disorganization of the splenic structure. H & E, × 70.

Fig. 6. Control mouse; spleen lymphatic nodule stained with the silver impregnation method to visualize reticulin fibers. Fibers are mostly localized at the periphery of the nodule. × 176.

Fig. 7. Spleen section of a mouse inoculated with Passage D extract and sacrificed 2 months later. The section is stained with the silver impregnation method. The fibers of reticulin form a network at the center as well as at the periphery of the disorganized nodule. × 176.

Fig. 8. Electron micrograph showing an infected plasmocyte (P); 2 viruses are budding along its plasma membrane. An endocytic vacuole containing some virus particles is also visible inside a reticular cell (R). × 35,910.

Fig. 9. Electron micrograph showing a virus budding. Its 3 different shells are readily visible. × 215,460.

Fig. 10. Electron micrograph of an immature type C particle showing the electron-lucent nucleoid. × 215,460.

Fig. 11. Electron micrograph of a mature type C particle showing an electron-dense nucleoid. The envelope exhibits a typical unit membrane structure. × 266,760.

Fig. 12. Electron micrograph of a cytoplasmic portion of a plasma cell. A few viruses are budding from the smooth membrane limiting the cytoplasmic vacuole. × 66,690.

Figs. 13 to 16. Electron micrographs of 2 spleen plasmocytes containing intracisternal type A particles. Fig. 13, × 23,085; Fig. 16, × 18,724. Higher magnification of the 2 plasmocytes illustrate the structure of the type A particle; 2 concentric shells can be seen (Fig. 14 from Fig. 13 and Fig. 15 from Fig. 16). Figs. 14 and 15, × 161,595.

Fig. 17. Electron micrograph of a spleen germinal center 8 days after inoculation with Passage D extract. Many C particles (arrow) are observed close to the infoldings of the plasma membrane of reticular cells. × 39,900.

Fig. 18. Electron micrograph of spleen white pulp, 7 days after inoculation with Passage D extract. An infected reticular cell shows a virus budding. × 10,260. Inset a, higher magnification of the budding particle. × 30,780.

Fig. 19. Electron micrograph of spleen white pulp, 1 day after inoculation with Passage D extract. The small lymphocyte shows a virus budding from its plasma membrane (arrow); × 18,810. a, higher magnification of the budding particle. × 100,548.

Fig. 20. Electron micrograph of a splenic plasmocyte, 29 days after Passage D extract inoculation. Budding particles can be observed along the plasma membrane (arrow). × 27,330.

Fig. 21. Electron micrograph of a spleen white pulp 14 days after inoculation with Passage D extract. A virus is budding from the plasma membrane of a plasmocyte (arrow). × 14,563. a, higher magnification of the budding particle. × 93,366.

Fig. 22. Electron micrograph of a spleen white pulp, 6 days after inoculation with Passage D extract. A virus is budding (arrow) from the plasma membrane of an immunoblast cell (Ib). × 15,390.

Fig. 23. Electron micrograph of a mouse spleen, 29 days after inoculation with Passage D extract. Note the numerous cells pertaining to the plasmocytic line. × 7,302.

Fig. 24. Electron micrograph of an infected mouse lymph node. A virus is budding (arrow) from the plasma membrane of a plasmocyte (P). Note the enlarged cisternae of the endoplasmic reticulum. × 9,975. a, higher magnification of the budding particle. × 28,500.

Fig. 25. Electron micrograph of a lymph node 29 days after inoculation with Passage D extract. Viruses are budding (arrows) from 2 plasmocytes (P). Note the well-developed Golgi apparatus. × 15,390. Inset a, higher magnification of the budding particle. × 59,850. Inset b, higher magnification of the budding particle. × 50,160.
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