Morphogenesis of Two Immunologically Induced Mouse Lymphomas

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SUMMARY

Female BALB/c and DBA/2N mice were treated simultaneously with azathioprine and an antigen (LDH virus, tubercle bacteria, or bovine serum albumin). Experimental animals developed between 20 and 66% malignant lymphomas that were classified by light and electron microscopy as lymphoblastic types. The tumors developed in an atrophic thymus and subsequently spread to involve the remaining lymphoreticular and hemoreticular tissues, as well as nearly all other organs. Cytologically, the initial thymic tumor nodules did not differ from hyperplastic nodules of nonneoplastic lymphoblastic stem cells. Also, cells of a well-established tumor showed only slight differences from those of normal lymphoblastic stem cells, such as increased esterase activity and presence of cytoplasmic annulate lamellae. Virus particles (C-type) were identified by electron microscopy only in early tissue culture passages of the BALB/c lymphoma but not in the DBA lymphoma. All tumors were readily isotransplantable. Cell-free transplantation remained negative in all instances tried. The tumor grows in tissue culture as an established lymphoblast cell line.

INTRODUCTION

The induction of malignant lymphomas by interference with the immune response against a variety of antigens was recently demonstrated (5, 11). It appears that this lymphoma development is less dependent upon the oncogenic transformation of cells by viruses but is rather a consequence of simultaneous persistent antigenic stimulation and immunosuppression (7, 8). These conditions, leading to the appearance of malignant lymphomas in this experiment, are quite similar to conditions under which certain human lymphomas develop, for instance those after kidney allotransplantation and those in immune deficiency syndromes (6, 7, 18, 19, 23). Since the morphology of human lymphomas is well described but the initiating lesions are still poorly known, a systematic morphological investigation was carried out on the development of immunologically induced malignant lymphomas in mice as a background for the initiation of a careful search for comparative lesions in human pathology.

Received October 4, 1971; accepted December 3, 1971.

MATERIALS AND METHODS

Tumor Induction. By a method described previously (7, 11), 386 female, 6-week-old BALB/cAnN and DBA/2N mice were immunosuppressed with azathioprine and antigenically stimulated with LDV, tubercle bacteria, or BSA. The mice from the Animal Production Branch of the NIH were caged in groups of 6 and fed Purina laboratory chow and tap water ad libitum. Azathioprine (Imuran; Burroughs Wellcome and Co., Tuckahoe, N. Y.) was administered in drinking water at an average dose of 15 mg/kg/day. LDV, originally obtained from Dr. Abner L. Notkins at the National Institute of Dental Research, was harvested from the serum of CAF mice. Six weeks after BALB/c mice were infected with this virus, serum was drawn from them. This serum served for infecting the experimental mice at a median infectious dose of $10^{9.5}$. The infectious dose was calculated by the method of Reed and Muench (21). Infection of mice was achieved by a single i.p. injection of 0.1 ml of virus-containing mouse serum. Tubercle bacteria organisms (H37Ra; Difco Laboratories, Inc., Detroit, Mich.) were suspended in 1 ml of adjuvant, and 0.05 ml of this suspension was injected s.c. into the thigh at biweekly intervals. Both hind legs were used alternately. BSA (BSA Fraction V powder; Pentex, Inc., Kankakee, Ill.) was made up to a 10% solution in phosphate-buffered saline, pH 7.4, and 0.25 ml of this solution was injected i.p. every day during the entire experiment.

Light and Electron Microscopy. Complete autopsies, excluding the brain, were done on randomly chosen experimental mice at monthly intervals starting 1 month after initiation of the experiment and at biweekly intervals after the 3rd month. Samples taken from all organs were fixed in Zenker's solution and embedded in paraffin, and 5-µm sections were stained with hematoxylin and eosin, Giemsa, PAS, methyl green-pyronin Y, and Wilder's reticulum stain. Blood smears and imprints from lymph nodes and spleen were treated with May-Grünwald-Giemsa stain.

In addition, selected tissue specimens were fixed in 3% buffered glutaraldehyde solution in Sorenson's buffer, postfixed with osmium tetroxide, and embedded in Epon. Ultrathin sections were cut on an LKB Ultratome, stained with uranyl acetate and lead citrate, and examined in a Siemens-Elmiskop IA electron microscope with a double condenser and a 50-µm objective aperture. An accelerating voltage of 80 kV was used.

The abbreviations used are: LDV, lactic dehydrogenase-elevating virus; BSA, bovine serum albumin; PAS, periodic acid-Schiff.
Histochemistry. The following 6 reactions were used for cytochemical characterization of lymphoma cells on selected imprints from tumor nodules: PAS (14), Sudan black B (17), nonspecific esterase (12), peroxidase (4), acid phosphatase (3), and alkaline phosphatase (2). Imprints from normal lymph nodes of BALB/c and DBA mice served as controls.

Transplantation Studies. Small pieces of tissue, approximately 1 cu mm, taken from different sites (thymus, lymph nodes, and spleen) were transplanted s.c. into the lateral abdominal wall of isogenic female mice not more than 6 months old. They were obtained under aseptic conditions and transferred by use of a No. 14 trochar attached to a syringe.

For cell-free transplantation, tumorous material from both BALB/c and DBA mice was homogenized under cooling in an ice bath (3000 rpm for 5 min in a Potter-Elvehjem glass homogenizer). After centrifugation of the debris (at 15,000 rpm for 10 min), the supernatant was pressed through a Millipore filter (pore size, 0.45 μm) and 0.1 ml of the resulting fluid was injected i.p. into 35 newborn and 35 isogenic 8-week-old mice of both strains.

Cell Culture Studies. Tumor tissue removed under aseptic conditions was minced by scissors in sterile phosphate-buffered saline (pH 7.4). After the addition of 0.25% trypsin with collagenase (2 ml/0.1 g of tissue) trypsinization was done, with stirring, for 1 hr. After being washed 3 times in phosphate-buffered saline, the cells were placed in tissue culture flasks containing Medium 1640 with glutamine and neomycin.

RESULTS

The incidence of malignant lymphomas was between 20 and 66%. Details are summarized in Table 1. The lymphomas were identified as 207K (BALB/c tumor) and F (DBA tumor).

Light Microscopy

The morphologies as well as the morphological developments of the 207K and F lymphomas were identical.

Table 1

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>No. of mice</th>
<th>Treatment</th>
<th>Antigen</th>
<th>Immunosuppression</th>
<th>Incidence of malignant lymphoma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>32</td>
<td>LDV</td>
<td></td>
<td>Azathioprine</td>
<td>32</td>
</tr>
<tr>
<td>BALB/c</td>
<td>32</td>
<td>TB</td>
<td></td>
<td>Azathioprine</td>
<td>20</td>
</tr>
<tr>
<td>BALB/c</td>
<td>64</td>
<td>BSA</td>
<td></td>
<td>Azathioprine</td>
<td>20</td>
</tr>
<tr>
<td>BALB/c</td>
<td>32</td>
<td>LDV</td>
<td></td>
<td>Azathioprine</td>
<td>0</td>
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<tr>
<td>BALB/c</td>
<td>32</td>
<td>TB</td>
<td></td>
<td>Azathioprine</td>
<td>0</td>
</tr>
<tr>
<td>BALB/c</td>
<td>46</td>
<td>BSA</td>
<td></td>
<td>Azathioprine</td>
<td>0</td>
</tr>
<tr>
<td>BALB/c</td>
<td>46</td>
<td>Azathioprine</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>DBA</td>
<td>34</td>
<td>BSA</td>
<td></td>
<td>Azathioprine</td>
<td>66</td>
</tr>
<tr>
<td>DBA</td>
<td>34</td>
<td>BSA</td>
<td></td>
<td>Azathioprine</td>
<td>0</td>
</tr>
<tr>
<td>DBA</td>
<td>34</td>
<td>Azathioprine</td>
<td></td>
<td></td>
<td>12</td>
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</tbody>
</table>

* TB, complete Freund’s adjuvant.
hemopoietic stem cells. These cells, however, progressively replaced the red pulp and, finally, nearly all of the follicles. However, even in late cases, follicular remnants often were still noted. In other spleens, the peripheral zone of the follicle or the entire follicle consisted of basophilic reticulum cells which spread subsequently to replace also most parts of the red pulp. Peyer's patches contained a solid population of basophilic reticulum cells.

Among other organs involved by infiltrates of these basophilic reticulum cells were liver, lungs, kidney, bone marrow, ovaries, heart, peripheral nerves, and the retroperitoneal space. In the liver, these cells populated the peribronchial and perivascular spaces contained nodules of basophilic reticulum cells that further invaded the interalveolar spaces (Fig. 11). Similarly, focal tumor spread occurred in the kidney from the perivascular spaces. The ovary often was nearly completely replaced by lymphoma cells, leaving only a few ova for organ identification. The aorta in several cases was sheathed by a large tumor mass. The bone marrow was completely replaced by tumor cells that in some instances also invaded the surrounding connective and muscle tissue (Fig. 12). There was no increase in reticulin fibers in areas of lymphoma growth. Blood smears during this period of tumor generalization showed a nearly monomorphous population of lymphoblasts.

The mice died quite emaciated, probably of respiratory failure secondary to large tumor masses in the mediastinum and in the lungs.

**Electron Microscopy**

In ultrathin sections of the tumor (Fig. 13), the majority of the cells resembled immature cells, especially those of the well-known lymphoblastic type. They contained large nuclei in which the chromatin was distributed quite evenly. The nucleoli were always very large. The amount of cytoplasm surrounding the nuclei was small and rich in ribosomes and contained few cytoplasmic organelles. Surprising was the high number of annulate lamellae in the cytoplasm of the blast cells (Fig. 14). A stroma consisting of fibroblasts was poorly developed. It separated layers of blast cells from each other.

**Histochemistry**

The cytochemical characterization of normal and tumor cells from the lymphoreticular tissues is given in Table 2. Positive reactions, when observed, were always cytoplasmic and finely granular. Normal lymphoreticular cells showing esterase activity usually met the morphological criteria of histiocytes, while in the tumor, also, lymphoblastic stem cells were esterase positive. The stainability of cells with Sudan black was faint, while quite a number of tumor cells (lymphoblasts) showed PAS-positive cytoplasmic granules, usually located in one circumscribed region close to the nucleus.

**Transplantation**

Transplantation of particulate tumor material into isogenic mice resulted in 100% takes in both BALB/c and DBA mice (i.e., both the 207K and the F lymphoma). The tumor remained isomorphous to the original, throughout 20 (F lymphoma) and 30 (207K lymphoma) generations. It grew rapidly by expansion, killing the emaciated recipients during the 4th week after transplantation. Cell-free transplantation did not result in tumor growth in any of the 140 mice.

**Cell Culture Experiments**

Tumor cells grew readily, leading to established suspension cultures. Histologically and by electron microscopy, the cells were identified as lymphoblasts (see "Electron Microscopy" and Fig. 15). They equaled morphologically the cells in the original tumor tissue. In the 1st passage, virus particles of the C type were observed in extracellular spaces. Their spikes, attached to the outermost viral envelope, were unusually well defined in ultrathin sections (Fig. 16). In addition to these cells growing in suspension, other cells attached to the glass and could be grown separately as cover-glass cultures. These cells by light microscopy were similar to fibrohistiocytic elements.

**DISCUSSION**

The first significant lesion to occur during the latent period of lymphoma development in our mice was an atrophy of the thymus. This atrophy per se did not differ from that observed after immunosuppressive treatment alone (9, 11), when it usually was not followed by tumor. No thymic cortical inversion was observed or described as preceding the appearance of a lymphocytic leukemia in AKR mice (15). Coinciding with or shortly following the thymic atrophy was a homologous atrophy of the lymph nodes, which in certain cases became subsequently more prominent in the paracortical region. This lesion suggests an insufficiency to initiate a cellular immune response secondary to the atrophy. A depressed cellular immune reaction indeed was demonstrated later, while the humoral immune response was not negatively influenced by coincident antigenic stimulation and immunosuppression (7, 8).

In the atrophic thymus, foci of immature stem cells develop while the atrophy of the lymph nodes is most prominent and while the cellular immune response is completely negative. The subcapsular region of the thymus where these cell nests are first noted is the area of normal cortical cell proliferation during thymic development. The type of cell in these foci

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**Table 2**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Tumor&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Normal cells&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS</td>
<td>(+) — (+++)</td>
<td>(+)</td>
</tr>
<tr>
<td>Sudan black</td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>Esterase</td>
<td>(+) — (+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup> —, negative; (+), occasional slightly positive cells; (+) — (+), few slightly positive cells; +, few positive cells; ++, several markedly positive cells.

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equals morphologically normal lymphoblasts. These observations suggest that this focal stem cell proliferation may be an attempt for thymic regeneration and for reconstitution of the cellular immune response. However, thymic regeneration under normal conditions—for instance, after partial surgical excision—is not likely to occur (1). The focal subcapsular cell proliferation in the thymus may reflect, therefore, an alteration in the proliferative capacity in this organ, although no cytologically abnormal cells are yet observed. This picture is completely different from the focal cell proliferation in the thymic medulla as observed by Hanna (M. G. Hanna, Jr., personal communication) to precede leukemia development induced by Gross leukemia virus. Since the lymphoblastic stem-cell proliferation in the thymic cortex of our mice carries the thymus-specific θ antigen (8), it is of considerable interest to know the type of the medullary cells described by Hanna; perhaps these are θ-negative T- or even B-cells (thymic dependent or bone marrow derived).

Subsequently, lymphoblastic stem cells from the thymic cortical region spread to replace the entire thymus and invade without apparent barrier the adjacent neck tissue. Also, the peripheral lymphoreticular tissues are progressively replaced by these cells. Although this cell spread resembles metastasis, the possibility cannot be excluded that, up to a certain limit, the normal mechanism of circulation of thymic cells with homing in certain peripheral lymphoreticular organs is present. This, indeed, may be suggested by early findings in the postcapillary venules (Fig. 8). The later entire replacement of these organs by lymphoblastic stem cells and their invasion into parenchymatous organs and connective tissue strongly suggest a malignant neoplastic spread by invasion, destruction, and metastasis. Cytological and cytohistochemical details at this time still reveal cells in the lymphoma resembling normal lymphoblastic stem cells (13, 22). The lymphoma cells differ from these only quantitatively in showing increased esterase activity and PAS-positive cytoplasmic granules. The meaning of annulate lamellae in the cytoplasm of the mouse lymphoma cells as also seen in other lymphomas, including Burkitt's tumor (see Ref. 20, Fig. 4), is not clear.

In spite of an extensive search, virus particles characteristic of the LDV were found in neither the original tumor tissue nor the cultured cells. The only virus particles seen were those of the C type. They were found in the 1st passage of the lymphoma 207K. Other virus particles, i.e., B-particles and intracytoplasmic A-particles, known to be a common contaminant in DBA mice, or intracytasternal A-particles, frequently seen in DBA and BALB/c mice, were not observed in the material studied.

Despite the absence of striking qualitative morphological differences between normal lymphoid cells and the proliferating cells in this experiment, it must be assumed that functionally our lymphoma cells differed from normal cells. The invasive and destructive growth pattern and the transplantation and cell-culture characteristics suggest a transformed, i.e., a neoplastic cell. No method used in this experiment, however, allows us to speculate at which step in the course of lymphoma formation this transformation to cancer occurred. Despite the fact that there appears to be no involvement of oncogenic viruses in this experiment, as judged from cell-free transfer studies and immunofluorescence investigations (8), future additional search for viral antigenic expression on the surface of the lymphoma cells may lead to further elucidation. This may be especially so since these transformed cells usually support viral replication, and viruses such as the C-type particle, for instance, are widespread contaminants in mouse colonies (16).

From the above descriptions it becomes clear that the morphological evolution of a lymphoreticular cancer from an inconspicuous hyperplastic nodule to a clear-cut invasive and destructive neoplasia may occur gradually. This accounts for the considerable difficulty in some hyperplastic human lymph nodes to establish or reject the diagnosis of a neoplastic disease. This was demonstrated in another study about lymphoreticular abnormalities in Chediak-Higashi disease (10), and it is certainly known also to every surgical pathologist confronted with a lesion suggestive of early Hodgkin's disease. Foci of immature stem cells in atrophic lymphoreticular tissues, however, should catch the attention of the morphologist and lead to a careful follow-up of the patient.

This study, combined with the immunological investigations cited (8), also shows that a malignant lymphoma may develop as a single focus in 1 organ and subsequently spread like metastases, as do other tumors. It therefore may be curable by removal of the primary focus before metastatic generalization has occurred. However, if the lymphoreticular atrophy or dysfunction underlying the development of the neoplastic disease is not reversible, the repeated occurrence of malignant lymphomas appears unavoidable.

ACKNOWLEDGMENTS

We thank Miss Duran Harris and Mr. B. Elliott, Jr., for technical assistance and Mr. Ralph L. Isenburg and Mr. Douglas Jones for photography. We are grateful to Dr. A. J. Dalton for stimulating discussions.

REFERENCES

Morphogenesis of Immunologically Induced Lymphomas


Fig. 1. Thymus of a female DBA mouse at 3 months of treatment with azathioprine and BSA. Note the marked loss of small lymphocytes, causing severe cortical narrowing. H & E, X 25.

Fig. 2. Thymus of a female DBA mouse at 3.5 months of treatment with azathioprine and BSA. Note focal proliferation of stem cells in atrophic cortex. H & E, X 150.

Fig. 3. Higher magnification of thymic cortical stem cell nodules. H & E, X 375.

Fig. 4. Lymph node of a female DBA mouse at 4 months of treatment with azathioprine and BSA. Note marked homologous atrophy. H & E, X 50.

Fig. 5. Malignant lymphoma replacing thymus and invading perithymic neck tissue in a female DBA mouse at 5 months of treatment with azathioprine and BSA. H & E, X 10.

Fig. 6. Starry-sky pattern of thymic lymphoma shown in Fig. 5. H & E, X 375.

Fig. 7. Lymph node of a female DBA mouse at 4 1/2 months of treatment with azathioprine and BSA. Note loose aggregates of stem cells in peripheral sinus. H & E, X 375.

Fig. 8. Postcapillary vein of in guinal lymph node in female DBA mouse at 4.5 months of treatment with azathioprine and BSA. Note "homing" of lymphoblastic stem cells along vascular endothelium. H & E, X 675.

Fig. 9. Spleen of a female DBA mouse at 5 months of treatment with azathioprine and BSA. Note subcapsular stem cell proliferation. H & E, X 150.

Fig. 10. Liver of a female DBA mouse at 6 months of treatment with azathioprine and BSA. Note dense population of lymphoma cells in sinusoids and perportal spaces. H & E, X 150.

Fig. 11. Lung of a female DBA mouse at 7 months of treatment with azathioprine and BSA. Note dense lymphoma cuffs surrounding vessels. H & E, X 150.

Fig. 12. Spine of a female DBA mouse at 8 months of treatment with azathioprine and BSA. Note complete replacement of hemopoietic bone marrow by lymphoma cells, and extensive invasion by lymphoma of adjacent soft tissues. H & E, X 150.

Fig. 13. Lymphoma F occurring in a DBA mouse consisting of blast-like cells. Large nuclei contain chromatin in its diffuse state. Nucleoli are well developed. Cytoplasm is rich in ribosomes, but cytoplasmic organelles are few. X 6200.

Fig. 14. Annulate lamellae (center of illustration) were observed frequently in the cytoplasm of the tumor cells. N, nucleus. X 60,000.

Fig. 15. Cells of lymphoma 207K (BALB/c mouse) grown in culture, 10th passage. The cells have the characteristics of lymphoid cells and exhibit well-developed Golgi zones (GZ) often surrounded by mitochondria. X 14,750.

Fig. 16. In the primary culture of lymphoma 207K virus particles of the C type were regularly seen. Two immature particles are illustrated that show clearly the electron-dense inner shell and the opaque intermediate shell. Note the relatively wide border of spikes around the particles. Note also the spikes around the obliquely cut virus particles (arrow). X 150,000.
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Cancer Res 1972;32:573-582.

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