MENINGEAL LEUKEMIA emerged as an important complication of acute lymphoid leukemia when improved therapeutic strategies increased the remission rate and prolonged survival. Meningeal prophylaxis based on skull irradiation, intrathecal chemotherapy, or both has reduced the incidence of meningeal leukemia to less than 10%. Morbidity and intellectual disabilities may be associated with these treatments. A better understanding of the process by which leukemic cells enter the brain may lead to an improved management of meningeal leukemia and, potentially, reduce the risk of overtreatment and of secondary complications.

Numerous studies on humans and animals have disclosed several features of meningeal leukemia, although little is known about the sequence of events through which the meninges are colonized by malignant lymphoblasts. Entry into the central nervous system (CNS) via the systemic circulation or from the skull bone marrow was always replaced by proliferating lymphoblasts. The difficulty of setting up an appropriate experimental model with human leukemic cells has represented a major complication of acute lymphoid leukemia when improved therapeutic strategies increased the remission rate and prolonged survival.

We have recently shown that nude (nu/nu) mice additionally immunosuppressed by splenectomy, sublethal irradiation, and treatment with antiasialo GM1 antiserum (SIA-nu/nu mice) have no detectable natural killer activity and allow the growth of human malignant lymphoblasts. We show here that all SIA-nu/nu mice engrafted intravenously with $5 \times 10^8$ malignant lymphoblasts originally derived from a child with T-cell acute lymphoblastic leukemia (PF382) and from a boy with a T-cell lymphoma (ST-4) develop lethal meningeal leukemia and die within 35 days. Histologic examination of moribund SIA-nu/nu mice showed that vertebral and skull bone marrow was always replaced by proliferating human T lymphoblasts. From the spinal canal, lymphoblasts spread to the meninges, causing hind leg paralysis. Leaving the skull, they permeated the meninges and then invaded the nervous parenchyma. This efficient and reproducible experimental model may be suitable for experimental studies on the pathogenesis of meningeal leukemia.

MATERIALS AND METHODS

Mice. Five-week-old female nu/nu mice on Swiss background were purchased from Charles River Laboratories (Calco, Italy) and allowed to rest for 1 week before any treatment. They were fed and maintained under specific pathogen-free conditions, and received sterilized food pellets and tap water ad libitum. Mice were splenectomized as previously described in detail and received total body, sublethal irradiation of 4.5 Gy from a $^{137}$Cs source providing a dose rate of 0.5 Gy/min 3 days before tumor challenge. Forty-eight and 24 hours before this challenge, they were additionally injected IV with 0.2 mL of a 1/10 dilution in phosphate-buffered saline (PBS) of antiasialo GM1 antiserum (Wako Chemicals GmbH, Dusseldorf, Germany; batch PDG9536) and referred to as SIA-nu/nu mice.

Malignant lymphoblasts. PF382 lymphoblasts were originally derived from the pleural exudate of a 6-year-old boy with T-cell lymphoblastic leukemia in relapse, and maintained as an in vitro cell line. ST-4 lymphoblasts were from a lymph node biopsy of a 12-year-old boy with a malignant T-cell lymphoma (convoluted type) currently alive and in prolonged complete remission.

Lymphoblast engraftment. PF382 or ST-4 malignant T lymphoblasts ($5 \times 10^8$) in 0.3 mL of Hanks' Balanced Salt Solution (HBSS) were injected IV into the tail vein of SIA-nu/nu mice, which were then inspected daily for signs of systemic involvement and spinal cord symptoms. Moribund mice were killed in extremis. Each experiment was performed independently four times using groups consisting of five mice. Because they gave homogeneous results, data are shown as mean $\pm$ SEM.

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RESULTS

Clinical course. By the day 25 to 30 after IV challenge with malignant human lymphoblasts, SIA-\textit{nu}/\textit{nu} mice presented anorexia, hunched posture, lethargy, and weight loss. A gait disturbance and signs of hind limb weakness become evident first in mice challenged with ST-4 lymphoblasts and later in those challenged with PF382 lymphoblasts. A marked leukopenia with relative neutrophilia but no leukemic blasts was found on differential cell count of smears of peripheral blood taken at weekly intervals after challenge from asymptomatic mice. Occasional leukemic blasts (1\% or less) were found when mice displayed initial neurologic signs. About 48 hours after the appearance of overt neurologic symptoms, mice become moribund and were killed. No neoplastic foci were ever found on histologic examination of lung and kidney from all of these moribund mice. Liver infiltration by PF382 cells was found in only one case (Fig 1A). By contrast, extensive bone marrow and CNS infiltration was always evident (Table 1).

Spine and medulla. In mice challenged with ST-4 cells, the vertebral bone marrow was replaced by rapidly proliferating lymphoblasts (Fig 1B). When a high local concentration was reached, lymphoblasts broke through the nutrient foramen and colonized muscles and/or meninges, and eroded the trabeculae of the vertebrae (Fig 1C and D). The meningeal infiltration was epidural and only later subdural, without lesions of the pia mater. Therefore, the spinal cord was never directly infiltrated, and hind leg paralysis was due to compression. Whereas ST-4 uniformly infiltrated the vertebral bodies, PF382 lymphoblasts were mainly located in the lumbar area. This distinct infiltration pattern explains why mice injected with ST-4 lymphoblasts became paralyzed earlier.

Encephalic area. In the initial experiments, the brains of SIA-\textit{nu}/\textit{nu} challenged mice were extracted before being processed for histologic observation. However, it became evident that this maneuver hampers correct evaluation, because it alters the meninges structure and brain-skull anatomical connections, and may lead to the loss of lymphoblast clusters attached to the brain surface (Fig 2).

When the whole cranium was decalcified and processed for histology, it was evident that lymphoblast infiltration primarily affects the skull bone marrow. Infiltration was not uniform, with the temporal bone being most frequently and massively infiltrated, followed by the sphenoid. Meningeal infiltration and extension of the leukemic infiltrates into the brain parenchyma follow direct transit of skull-infiltrating lymphoblasts. These lymphoblasts progressively erode the junction of the squama with the petrosa portion of temporal bone (Fig 2A and B) and the sphenoid (Fig 2C), invade the dura mater and the subarachnoid spaces (Fig 2C) with permeation of the pia mater, and infiltrate the Virchow-Robin spaces and the nervous parenchyma late (Fig 2D, E, and F).

This progression pattern was almost identical after both ST-4 and PF382 lymphoblast challenge. Minor differences were noted in the distribution and extension of bone marrow infiltration, and meningeal spreading as PF382 lymphoblasts showed more extensive cerebral infiltration with areas of necrosis.

Malignant lymphoblasts from SIA-\textit{nu}/\textit{nu} mice. The immunoperoxidase technique was used to stain spinal cord and encephalic area sections with an antihuman HLA A, B, C MoAb, as previously described. The large infiltrating cells were always positive. Flow cytometry and cytogenetic analysis on lymphoblasts recovered from bone marrow and meninges cultures displayed no significant modulation of surface and karyotype markers when compared with ST-4 and PF382 lymphoblasts before engraftment (data not shown).

DISCUSSION

Splenectomy, irradiation, and injection of antiasialo GM1 antiserum required for SIA-\textit{nu}/\textit{nu} mice are simple procedures that can be easily standardized and quickly performed. The resulting SIA-\textit{nu}/\textit{nu} mice are healthy, long-living animals that display no detectable NK activity in the blood and the spleen, clear human malignant lymphoblasts very slowly, and allow their local growth and systemic dissemination.

The pattern of dissemination of malignant human T lymphoblasts injected IV into SIA-\textit{nu}/\textit{nu} mice enables a model of selective leukemic invasion of the CNS to be created. The effectiveness and reproducibility of CNS invasion makes it a suitable model for experimental studies on meningeal leukemia and on its pathogenesis.

In SIA-\textit{nu}/\textit{nu} mice, both PF382 and ST-4 human lymphoblasts injected IV localize first in the bone marrow and then disseminate to the surrounding tissues, and to the CNS in particular, whereas a hematologic dissemination was never found.
In SIA-\(nu/nu\) mice, the spread and progression pattern of PF382 and ST-4 cells is remarkably consistent. However, in untreated \(nu/nu\) mice, as well as in those only splenectomized or sublethally irradiated, ST-4 and PF382 lymphoblasts either do not grow or they vary to a great extent their growth and dissemination pattern.\(^{16,17}\) Moreover, when PF382 lymphoblasts are injected in SIA-\(nu/nu\) mice treated with a concentration of antiasialo GM1 antiserum, only half of that used in this study, their growth pattern is markedly different. They first invade the liver and kidney, whereas CNS localization is an occasional and late event.\(^{17}\) Scid/scid mice have T- and B-lymphocyte defects, but a normal NK activity. They allow the growth and spread of many childhood acute lymphoblastic leukemias, but the CNS is never primarily affected.\(^{14}\) In these mice, PF382 and ST-4 lymphoblasts form a tumor when injected subcutaneously, but do not spread after an IV challenge (data not shown). As a whole, these findings show that the addition of antiasialo GM1 treatment to other major immunodeficiency statuses as well as the dose used are important in determining the spread of malignant lymphoblasts and getting a selective CNS invasion.

One of the most evident effects of antiasialo GM1 antiserum is the abolition of the NK activity of recipient mice.

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**Table 1. Features of Clinical Course and Lymphoblast Localization in SIA-\(nu/nu\) Mice Challenged With PF382 and ST-4 Human T-Malignant Lymphoblasts**

<table>
<thead>
<tr>
<th>Mice Challenged IV With</th>
<th>Meningeal Leukemia Takes/Total Challenged Mice</th>
<th>Spread of Malignant Lymphoblasts</th>
<th>Days When Mice Become Moribund</th>
</tr>
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<tbody>
<tr>
<td>PF382</td>
<td>20/20</td>
<td>Lung* 0 26-32</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Liver* 5</td>
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<td></td>
<td></td>
<td>Kidney* 0</td>
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<td></td>
<td></td>
<td>Bone marrow* 100</td>
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<td></td>
<td></td>
<td>Meninges* 100</td>
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<tr>
<td></td>
<td></td>
<td>Blood (&gt;1/100)†</td>
<td>0</td>
</tr>
<tr>
<td>ST-4</td>
<td>19/19</td>
<td>Lung* 0 23-27</td>
<td></td>
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<td>Liver* 0</td>
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<td></td>
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<td>Blood (&gt;1/100)†</td>
<td>0</td>
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*Presence of histologically detectable foci of malignant lymphoblasts.

†Lymphoblasts/peripheral blood leukocytes.
mice. This produces a major delay in the clearance of human and murine lymphoblasts from nu/nu mice and increases the growth and dissemination of many tumors. Thus, it is likely that NK cells play a direct role in the spread of malignant lymphoblasts and the appearance of meningeal leukemia. However, in addition to NK cells, other asialo GM1+ cells responsible for natural resistance to malignant lymphoblasts can be affected by the amounts of antiserum used. The role of natural immune reactivity mechanisms in the pathogenesis of meningeal leukemia is a subject for future research.

The sequence of events behind leukemic infiltration of the meninges and brain is debated. Our histologic findings strongly suggest that the primary event is lymphoblast infiltration of the bone marrow, whereas meningeal leukemia is a subject for future research.

Because only the PF382 and ST-4 lymphoblasts have been used in these experiments, no general conclusions can be drawn. However, their spreading in SIA-nu/nu mice may be representative of malignant T lymphoblasts, because PF382 and ST-4 cells display a similar spreading pattern, but differ in membrane markers and stage of maturation along the T-cell differentiation lineage, and were obtained from children with a very different clinical course. Admittedly, injection of human lymphoblasts into immuno-deficient and further experimentally immunosuppressed xenogeneic hosts results in an artificial model. The extent to which the data obtained mimic the situation occurring in humans needs to be carefully considered. The possibility that the spreading pattern observed is the result of the peculiar interactions between human and murine homing receptors cannot be ruled out. Nevertheless, our data fit in well with the findings of Thomas and Bleyer concerning the direct spread of both syngeneic murine and human leukemic cells from the skull bone marrow to the brain and nervous system in meningeal leukemia. If human leukemic cells primarily infiltrate the CNS by direct spread from cranial bone marrow, craniospinal radiotherapy, which eradicates malignant lymphoblasts from cranial and vertebral bone marrow, may be a more effective way of preventing such infiltration than systemic chemotherapy. Unfortunately, it is associated with significant adverse neuropsychologic and neuroendocrine effects, including impairment of intellectual function. However, in SIA-nu/nu mice, malignant lymphoblast infiltration shows remarkable differences between skull bones. If the SIA-nu/nu data truly delineate some aspects of human meningeal leukemia, the identification of bones more at risk than others in children with acute leukemia could lead to the establishment of a maximally effective and minimally damaging form of prophylaxis.

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Growth and spread of human malignant T lymphoblasts in immunosuppressed nude mice: a model for meningeal leukemia

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