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 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.

Previous work has 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 × 10⁶ malignant lymphoblasts originally derived from a child with a 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.

GROWTH AND SPREAD OF HUMAN MALIGNANT T LYMPHOBLASTS IN IMMUNOSUPPRESSED NUDE MICE: A MODEL FOR MENINGEAL LEUKEMIA

By Federica Cavallo, Marco Forni, Carlo Riccardi, Antonio Soleti, Francesco Di Pierro, and Guido Forni

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 ¹³⁷Cs 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 ½ 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 × 10⁶) 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 progression pattern in 100% of SIA-nu/nu mice.

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results, as determined by the Snedecor and Irvin test,20 the data were cumulated.

**Blood smears.** At weekly intervals after challenge and when mice displayed initial neurologic signs, peripheral blood smears of all challenged SIA-\(\text{nu}/\text{nu}\) mice were performed through punctures of the tail lateral vein and routinely stained for differential count. At least 300 leukocytes were counted for each mouse.

**Histology.** Thoracic and abdominal organs of all mice were inspected, extracted, fixed in buffered formalin, and routinely processed for histologic examination.19 To maintain the anatomical connection among bones and meninges, the head was skinned, fixed for 48 hours, completely decalcified in Osteodec (Farben: Bio-Optica, Milan, Italy) for 24 to 48 hours, and then sliced with frontal cuts in six to seven parallel slices 2 to 3 mm thick, including cranial bones and cerebrum. A transversal section was cut from the dorso-lumbar region of the decalcified spines, whereas the two remaining halves were cut in half sagittally. The tissues were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Some tissues were also snap-frozen and immunostained with mouse monoclonal antibody (MoAb) to HLA A, B, C antigens (TEC-HLA ABC; Sorin, Saluggia, Italy) using an avidin-biotin procedure (ABC, Vectastain; Vector, Burlingame, CA), as previously described.17

**Recovery of malignant T-blasts.** Fragments of skull bones were placed in complete medium in 24-well plates (Nunc, Roskilde, Denmark). The lymphoblasts recovered were stained with fluorescein isothiocyanate (FITC)-conjugated anti-HLA A, B, C, CD1 and CD7 murine MoAb (Sorin) and analyzed with a FACScan flow cytometer (Beckton Dickinson, Milan, Italy). Karyotype analysis was performed as previously described.19

**RESULTS**

**Clinical course.** By the day 25 to 30 after IV challenge with malignant human lymphoblasts, SIA-\(\text{nu}/\text{nu}\) 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-\(\text{nu}/\text{nu}\) 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-\(\text{nu}/\text{nu}\) 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.15 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-\(\text{nu}/\text{nu}\) mice are simple procedures that can be easily standardized and quickly performed. The resulting SIA-\(\text{nu}/\text{nu}\) 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.15-17

The pattern of dissemination of malignant human T lymphoblasts injected IV into SIA-\(\text{nu}/\text{nu}\) 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.15-17

In SIA-\(\text{nu}/\text{nu}\) 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.
Fig 1. Growth of PF382 and ST-4 human lymphoblasts in the liver and spine. (A) Liver: Evident lymphoblast foci were found in only 1/20 mice challenged with PF382 lymphoblasts. (B, C, and D) Spine: Infiltration by ST-4 lymphoblasts that colonize vertebral bone marrow (b) and meningeal spaces. (B) The epidural infiltrate is evident at vertebral body level, whereas it disappears at the vertebral disk level (arrow). (C) From the vertebral bone marrow lymphoblasts break through the nutrient foramen of the bone (arrow) to the meningeal spaces (arrow), whereas the medulla (m) is not invaded. (D) Erosion of the vertebral bone table and spilling of the lymphoblasts into the epidural spaces (black arrow) and, on the opposite site, invasion of skeletal muscles (white arrow). Original magnification: A and B, ×10; C and D, ×25.

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. Moreover, when PF382 lymphoblasts are injected in SIA-nu/nu mice treated with a concentration of antiasialo GM1 antiseraum, 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. 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. 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 antiseraum is the abolition of the NK activity of recipient

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 With</th>
<th>Meningeal Leukemia Takes/Total Mice</th>
<th>Spread of Malignant Lymphoblasts</th>
<th>Days When Mice Become Moribund</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV With</td>
<td></td>
<td>Organ</td>
<td>% of Mice</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lung*</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Liver*</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney*</td>
<td>0</td>
</tr>
<tr>
<td>PF382</td>
<td>20/20</td>
<td>Bone marrow*</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Meninges*</td>
<td>100</td>
</tr>
<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*</td>
<td>0</td>
</tr>
<tr>
<td></td>
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<td>Liver*</td>
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<td>Meninges*</td>
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<tr>
<td></td>
<td></td>
<td>Blood (&gt;1/100)†</td>
<td>0</td>
</tr>
</tbody>
</table>

*Presence of histologically detectable foci of malignant lymphoblasts.
†Lymphoblasts/peripheral blood leukocytes.

Fig 2. Meningeal invasion of the encephalic area by PF382 and ST-4 lymphoblasts. (A and B) Bone marrow infiltration of the temporal bone by PF382 lymphoblasts. Slight (A) (arrows) and massive (B) infiltration of the meningeal spaces by lymphoblasts coming from the bone marrow. (C) ST-4 lymphoblasts coming out from the sphenoid bone(s) form a large meningeal deposit in the base of the skull. Interestingly, the biconvex shape of the infiltrate is reminiscent of a subdural hemorrhage. Pia mater and cortex (c) are not yet infiltrated. (D, E, and F) Progressive phases of brain parenchyma invasion by PF382 lymphoblasts. (D) Initially, the meningeal leukemic infiltration is confined to arachnoidal spaces. No violation of pia mater is yet present. Virchow-Robin spaces are not invaded by lymphoblasts (white arrow). (E) Initial permeation of pia mater. Few lymphoblasts are invading the superficial cortex. Virchow-Robin spaces are both noninvaded (white arrow) and invaded (black arrow). (F) Diffuse infiltration of the superficial cortex and invasion of Virchow-Robin spaces (black arrows). Original magnification: A, B, and C, ×25; D, E, and F, ×100.
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 and brain invasion occurs later. Confirmation of this succession is provided by the observation of a high blast proportion in the meninges right beside the sites where the passage from the bone compartment to the surrounding areas is evident. However, this passage must not be regarded as a sign of tropism towards the CNS on the part of leukemic blasts, because they equally spread from the marrow towards the muscles. In fact on reaching the brain from the skull malignant blasts proliferate to the point where the pressure they build up drives them first through the nutrient foramen and then through holes they themselves erode in the bone table. The escaping cells accumulate in the epidural space and are again driven by compression to permeate the dura mater and infiltrate the leptomeninges, but do not occupy the Virchow-Robin spaces. When this infiltration becomes massive, they traverse and disrupt the pia mater to reach the brain parenchyma. Virchow-Robin space invasion becomes evident at this stage only. Therefore, infiltration of the meninges is not the direct outcome of invasion of the walls of their veins. Systemic circulation of malignant blasts serves only as the means whereby they reach the marrow. The lack of massive blood invasion by lymphoblasts and the pattern of progression of the CNS invasion with the late invasion of Virchow-Robin spaces make it unlikely that a major entry of malignant lymphoblasts into the CNS significantly occurs via the systemic circulation. It would not have been possible to establish this sequence of events had the brain been removed from the skull, and in the absence of in toto sections.

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 immunodeficient 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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