The study of central nervous system (CNS) leukemia has been hampered by the lack of a suitable animal model. We report that severe combined immunodeficiency (SCID) mice invariably develop rapidly progressive fatal CNS leukemia within 3 weeks after intravenous injection of NALM-6 pre-B acute lymphoblastic leukemia (ALL) cells. Colonization of the dura mater and subarachnoid space, usually of the distal spinal cord with occasional extension into the Virchow-Robin spaces of blood vessels subjacent to the meninges, followed involvement of bone marrow in the skull, vertebrae, and, occasionally, the appendicular skeleton. Occult CNS leukemia was detectable by polymerase chain reaction amplification of human DNA as early as 8 days postinoculation of leukemia cells. We used this in vivo model of human CNS leukemia to examine the therapeutic efficacy and toxicity of intrathecally administered B43 (anti-CD19)-pokeweed antiviral protein (PAP), an anti-B-lineage ALL immunotoxin directed against the pan-B-cell antigen CD19/Bp95. Intrathecal therapy with B43 (anti-CD19)-PAP immunotoxin at nontoxic dose levels significantly improved survival of SCID mice and was superior to intrathecal methotrexate therapy.

Intrathecal CNS therapy with B43 (anti-CD19)-pokeweed antiviral protein (PAP) immunotoxin, a new anti-ALL agent that does not cross the blood-brain barrier, was more effective in achieving long-term event-free survival (EFS) than systemic therapy with the same agent, suggesting that seeding of BM and other extramedullary sites by blasts from the CNS plays a major role in progression of leukemia in this model system.

The SCID mouse model of human CNS leukemia described in this report is devised to provide a basis for future comparative studies of the toxicity and efficacy of new CNS therapy regimens in B-lineage ALL. A major finding of clinical significance reported here is that leukemic blasts in the CNS can be effectively eradicated by weekly intrathecal administration of B43-PAP immunotoxin without side effects. These experiments provide a paradigm for the critical evaluation of a new CNS therapy regimen in B-lineage ALL.

MATERIALS AND METHODS

SCID mice. SCID mice were produced by specific pathogen-free (SPF) CB-17 SCID/SCID breeders in the American Association for the Accreditation of Laboratory Animal Care (AAALAC)-accredited Research Animal Resources Facility of the University of Minnesota (Minneapolis, MN). SCID mice were housed in microisolator cages containing autoclaved food, water, and bedding. Trimethoprim-sulfamethoxazole was added to the drinking water for a 3-day period each week.
Cells and inoculation. The previously described human NALM-6-UM1 (NALM-6) pre-B ALL cell line was maintained by serial passage in RPMI 1640 medium ( GibcoB Laboratories, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 50 μg/mL streptomycin, 50 IU/mL penicillin, 2 mmol/L L-glutamine, and 10 mmol/L HEPES buffer. Cells were cultured in tissue culture flasks at 37°C in a humidified 5% CO2 atmosphere. Before injection, cells were washed twice in phosphate-buffered saline (PBS) and resuspended in PBS at 2.5 × 10^7/mL or 5 × 10^7/mL. Inoculation was into the dorsal tail vein.

Drugs. B43-PAP immunotoxin was produced in the Immunotoxin Production Facility of the University of Minnesota Biotherapy Program, as previously described. Methotrexate (25 mg/mL) was obtained from Lederle Parenterals Inc (Carolina, Puerto Rico).

Intrathecal and systemic therapy for xenografted human CNS leukemia. Intrathecal injections were made into the lumbar space using a syringe with a 25-gauge needle, and 20 ng substance P (total volume, 1 μL) was coadministered to confirm the success of the intrathecal injections; substance P induces a caudally directed leukemic burden at the time of initiation of treatment. After the administration of B43-PAP, single IO- to 25-μg intrathecal doses were administered to Balb/c mice to further characterize the histologic lesions of the spinal cord.

Examination of SCID mouse tissues for xenografted human leukemia cells. At the time of death, necropsies were performed, and tissues, including BM and brain, of SCID mice were collected for evaluation of the human pre-B ALL leukemia burden. Tissues from mice that died or were euthanized by exposure to CO2 gas were fixed with 10% neutral buffered formalin by perfusion via the left ventricle or by immersion. The axial and appendicular skeleton, containing the CNS and BM, were subsequently decalcified in 10% formic acid for 48 hours, Representative portions of tissues, including multiple sections of all regions of the spinal column, were dehydrated and embedded in paraffin by routine methods. Six-micron, hematoxylin and eosin-stained sections were examined. For immunophenotypic studies, leukemic cells were dissociated from the leptomeninges of the brain by gently vortexing the whole brain in PBS in a test tube. BM suspensions were prepared by flushing the narrow cavities of long bones with PBS using a syringe with a 25-gauge needle. Other tissues were gently homogenized in PBS. Multi-parameter flow cytometric analyses using two-color immunofluorescence staining techniques and pairwise combinations of a selected panel of fluorochrome (fluorescein isothiocyanate (FITC) or phycoerythrin (PE))-labeled monoclonal antibodies (MoAbs) were performed on the cell suspensions, as previously described.

RESULTS

Human pre-B ALL cell line NALM-6 causes fatal CNS leukemia in SCID mice. Of 83 SCID mice challenged with an IV inoculum of 1 × 10^6 NALM-6 cells, 83 developed symptomatic CNS leukemia. The first clinical sign of CNS leukemia was a muscle weakness in the rear legs with a slight lowering of the lower back at a median of 32 days (mean ± SE, 33 ± 1 days; range, 26 to 58 days). Subsequently, mice developed a rapidly progressive paralysis of the rear legs, and at a median of 39 days (range, 36 to 56 days), they were no longer able to obtain food or water.

### Table 1. Kinetics of Colonization of SCID Mouse Tissues by NALM-6 Cells

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Assay</th>
<th>Days After Inoculation With 5 × 10^6 NALM-6 Cells</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>BM</td>
<td>PCR</td>
<td>-</td>
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<td></td>
<td>Histopathology</td>
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<td>Brain</td>
<td>PCR</td>
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<tr>
<td></td>
<td>Histopathology</td>
<td>-</td>
</tr>
<tr>
<td>Dura mater of the brain</td>
<td>PCR</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Histopathology</td>
<td>-</td>
</tr>
<tr>
<td>Leptomeninges of the brain</td>
<td>PCR</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Histopathology</td>
<td>-</td>
</tr>
</tbody>
</table>

Groups of three SCID mice were killed on the indicated days after inoculation with 5 × 10^6 NALM-6 cells, and assays were performed as detailed in Materials and Methods. PCR was performed on whole brain without dura mater. Histology of the CNS was performed on sections of the brain and spinal cord.

Abbreviations: PCR, PCR amplification of human β-globin gene sequences; ND, not done; +, detection by PCR or histopathology in three of three mice; -, absence of leukemic cells by PCR or histopathology in three of three mice.

* First day any animal in a group was positive.
because of paralysis. Some of these mice suffered a generalized tonic-clonic seizure followed by sudden death, whereas others either died without a preceding seizure or they were electively killed in moribund condition. Postmortem histopathologic studies showed that the CNS was extensively involved, with dense leukemic cell infiltrates in the subarachnoid space and the superficial perivascular Virchow-Robin spaces (data not shown). Large numbers of CD10\(^+\)CD19\(^+\)CD20\(^-\) human NALM-6 leukemia cells could be dissociated from the leptomeninges of the brain by gently vortexing the whole brain of paralyzed SCID mice in PBS in a test tube.

To further study the pathobiology of xenografted human CNS leukemia, SCID mice were inoculated in the dorsal tail vein with \(5 \times 10^6\) NALM-6 cells. Mice were observed daily, and groups of three mice were killed on days 1, 2, and 5 and at 3-day intervals from 8 days to 30 days postinoculation and subjected to pathologic analysis to assess their leukemia burden. Xenografted human leukemia cells were first detected in the BM, always starting in the skull or vertebrae. All mice had histologically detectable BM involvement in the skull or vertebrae and occasionally the appendicular skeleton by day 11 (Table I). NALM-6 cells in hematoxylin and eosin-stained sections of paraffin-embedded mouse BM were easily distinguished from murine hematopoietic cells. Leukemic cells were 10- to 13-\(\mu\)m in diameter, with oval to irregularly polygonal nuclei with smooth borders, finely stippled chromatin, and a single prominent eosinophilic nucleolus. They had scant, lightly basophilic cytoplasm and a high mitotic rate. Colonization of the dura mater and subarachnoid space, usually of the distal spinal cord, was observed as early as 20 and 23 days postinoculation, respectively, and colonization of both sites was a constant feature after 23 days (Table I, Fig 1). Histologically evident CNS involvement was limited to the dura mater and subarachnoid space, with occasional extension into the Virchow-Robin spaces of blood vessels subjacent to the meninges; no leukemic cells were seen in the Virchow-Robin spaces of blood vessels deep within the parenchyma of the CNS.

The kinetics of engraftment of NALM-6 cells in SCID mouse tissues was also examined by PCR analysis. Groups of three mice were killed on days 1, 2, and 5 and at 3-day intervals from day 8 to day 20, and their occult leukemia burden was evaluated by PCR amplification of human DNA, as described.\(^{17,18}\) As shown in Fig 2A, no PCR evidence of occult CNS leukemia was found in SCID mice at 1 to 5 days after inoculation of NALM-6 cells. By contrast, positive control SCID mice that died of disseminated leukemia with CNS involvement showed strong PCR positivity for human \(\beta\)-globin gene sequences in DNA specimens from BM and brain (Fig 2B). Similarly, PCR analysis of SCID mouse BM and brain invariably showed presence of human DNA at 8, 11, 14, 17, and 20 days (Fig 2C through E). Although histologically detectable engraftment of leukemia cells in skull or vertebral BM and meninges preceded engraftment in other sites, PCR analysis of human DNA detected leukemic cell engraftment in extraneural sites (eg, liver) as early as in the CNS and BM (Fig 2C).

Several features suggest that a major mechanism of colonization of the meninges by NALM-6 cells was by direct extension from the BM of the calvaria and vertebrae. (1) Colonization of the BM of the calvaria and vertebrae always occurred earlier in the course of disease than did colonization of the meninges. (2) Meningeal disease was always accompanied by leukemic involvement of the adjacent BM. (3)
Colonization of the endosteum occurred earlier than colonization of the dura mater, and colonization of the dura mater was always accompanied by involvement of the adjacent endosteum. (4) Colonization of the subarachnoid space was always accompanied by involvement of the adjacent dura, whereas the reverse was not always true. (5) Streams of leukemic cells could be seen entering the CNS from the BM by direct penetration through the cortex, along vascular channels or along nerve roots (Fig 3).

**Intrathecal B43 (anti-CD19)-PAP therapy is effective against xenografted human CNS leukemia in SCID mice.** Occult leukemia of the CNS was shown by PCR analysis of brains at the time of initiation of treatments (14 days after inoculation of 1 x 10^6 NALM-6 cells) in four of four SCID mice that were randomly selected at the time of assignment to treatment groups. Histologic examination of the CNS showed leukemic cells in one of six mice at this timepoint. All 23 saline-injected control mice became paralyzed and developed histologically demonstrable CNS and systemic leukemia. Intrathecal administration of methotrexate did not significantly increase survival of the SCID mice, and all 10 mice that received intrathecal methotrexate treatment died with extensive CNS leukemia at a median of 49 days (Table 2, Fig 4). Three mice treated with intrathecal injections of unconjugated B43 antibody (2.5 μg per dose) died of leukemia at 46, 52, and 59 days, respectively (data not shown). Similarly, all mice treated with weekly intraperitoneal B43-PAP injections died of disseminated leukemia at a median of 44 days (Table 2). The presence of xenografted NALM-6 cells in BM and/or CNS of SCID mice treated with normal saline, intraperitoneal B43-PAP, or intrathecal methotrexate was confirmed by PCR detection of human DNA (Fig 5A and B). Intrathecal administration of B43-PAP prolonged survival of SCID mice inoculated with NALM-6 leukemia cells (Table 2, Fig 4). The median survival time of mice administered weekly intrathecal doses of 2.5 μg B43-PAP (ie, 68 days) was significantly greater than the median survival time of the saline control group (ie, 41 days) or the methotrexate-treated group (ie, 49 days; \( P < .0001 \); Table 2). Two mice in the 2.5 μg intrathecal B43-PAP group were in good health at the end of the 111 day observation period and had no histologic evidence of leukemia in the CNS or BM. The human DNA contamination in the BM or brain of these mice appeared to be less than 0.001% (Fig 5A, C, and D). For each of the three intrathecal B43-PAP treatment groups receiving B43-PAP at 1.0, 2.0, or 2.5 μg per dose, four mice that died during the 120-day observation period were evaluated histologically. All 12 mice had disseminated leukemia with CNS involvement.

Systemic administration of B43-PAP does not provide an alternative to intrathecal B43-PAP therapy for xenografted CNS leukemia, as B43-PAP immunotoxin molecules are too large to cross the blood-brain barrier. As shown in Table 2, intraperitoneal administration of 2.5 μg B43-PAP, given at the same dose schedule as the intrathecal B43-PAP regimen, did not increase survival, indicating that intrathecal administration was the key factor in increasing survival time. Intraperitoneal administration of 20 μg B43-PAP in three equally divided consecutive daily doses on days 14, 15, and 16 after inoculation with NALM-6 cells significantly prolonged survival (median survival, 65 days; \( P < .02 \)), but all mice in this group died with CNS leukemia (Table 2).

There was no histologic evidence of neurotoxicity in the mice that received intrathecal B43-PAP doses up to 2.5 μg. However, all 10 mice that received intrathecal injections of 10 μg B43-PAP developed rear leg paralysis shortly after...
Fig 3. Penetration of human leukemia cells from BM into CNS. Caudal spinal column of a paralyzed SCID mouse showing streams of leukemia cells (arrows) penetrating from the vertebral BM space (BM) to the epidural space (ES). H&E stain; OM: (A), × 50; (B), × 200.

the onset of treatment (Table 2). All but one of these mice only developed signs of neurotoxicity after two or more injections of B43-PAP. All six histologically examined mice in this group had severe damage of the terminal sacral spinal cord (site of injection), characterized by severe necrosis of gray and white matter, as well as degeneration of tracts within the ventral nerve roots of this region (Fig 6). There were no morphologic lesions of the blood vessels. Neurotoxicity was the cause of paralysis in these mice, as no histologic or PCR evidence of CNS leukemia was found.

DISCUSSION

We report that the SCID mouse microenvironment permits the development of a rapidly progressive and invariably fatal CNS leukemia after inoculation with human NALM-6 pre-B ALL cells via a tail vein. Also provided is experimental evidence that intrathecal B43 (anti-CD19)-PAP immunotoxin therapy at a nontoxic dose level significantly improves survival of SCID mice with xenografted CNS leukemia.

The first histologic evidence of CNS leukemia in this model is often found in the distal spinal cord, preceding
involvement of the brain. The reason for this predilection is not known, but may be related to inoculation with the NALM-6 cells into the tail vein. There are anastomoses between abdominal veins and vertebral veins in other species, suggesting that these may be avenues of colonization of the CNS. Of interest is the finding that one avenue of colonization of the meninges of the brain and spinal cord appears to be direct extension from adjacent BM, as has been suggested by others. This may not be the consequence of specific tropism of these cells for the CNS, however, as invasion of the adjacent skeletal muscle from the BM also commonly occurred.

PCR amplification detected human DNA in the CNS of the SCID mice before histologically demonstrable proliferation of NALM-6 cells in the adjacent BM, suggesting that colonization of the CNS may also occur by a means other than direct extension from the marrow. These leukemic cells in the CNS may account for the eventual disseminated leukemia in mice treated only by intraperitoneal administration of B43-PAP, as seen in this and a previous study.

Intrathecal therapy for occult CNS leukemia with B43-PAP significantly prolonged survival of SCID mice inoculated 14 days previously with $1 \times 10^6$ NALM-6 pre-B ALL cells and resulted in the apparent cure of several mice. Intraperitoneal therapy with B43-PAP (20 $\mu$g total dose administered on 3 consecutive days) was not as efficacious as intrathecal therapy, as would be expected from therapy that does not pass the blood-brain barrier. However, it increased survival of SCID mice, suggesting that it eliminated many, if not all, accessible leukemic cells. PCR assay showed that human leukemic cells were present in the CNS of the SCID mice at the initiation of intraperitoneal treatment, suggesting that these cells may be the progenitors of the systemic leukemia found at the time of death in these mice.

Interestingly, mice treated intrathecally or intraperitoneally with $\geq 2.0 \mu$g B43-PAP and that died with leukemia early (less than 45 days) in the course of the study tended to have only leukemic involvement of the spinal cord and adjacent vertebral BM, whereas mice that died later (greater than 50 days) had disseminated disease with leukemic involvement of the spinal cord, BM, and viscera. Therefore, we speculate that (1) CNS leukemia causes paralysis that necessitates euthanasia before severe morbidity due to involvement of BM or viscera occurs, and (2) the CNS serves as a source of cells that seed extraneural sites. According to this hypothesis, incomplete elimination of leukemia cells in the CNS could have resulted in paralysis from CNS leukemia before dissemination of the disease, whereas late deaths from disseminated leukemia could have occurred if a small number of leukemic cells in the CNS survived therapy, leading to slow colonization of the CNS and migration to extraneural tissues before CNS involvement resulted in paralysis.

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**Table 2. Antileukemic Efficacy of Intrathecal B43 (anti-CD19)-PAP**

<table>
<thead>
<tr>
<th>Treatment Regimen</th>
<th>No. of Mice</th>
<th>Median EFS (d)</th>
<th>Cumulative Proportion Surviving Event-Free (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 d</td>
</tr>
<tr>
<td>NS it qwk x 4 wk</td>
<td>23</td>
<td>41</td>
<td>100</td>
</tr>
<tr>
<td>0.5 $\mu$g B43-PAP it qwk x 4 wk</td>
<td>9</td>
<td>42</td>
<td>100</td>
</tr>
<tr>
<td>1.0 $\mu$g B43-PAP it qwk x 4 wk</td>
<td>10</td>
<td>36</td>
<td>80</td>
</tr>
<tr>
<td>2.0 $\mu$g B43-PAP it qwk x 4 wk</td>
<td>10</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>2.5 $\mu$g B43-PAP it qwk x 4 wk</td>
<td>10</td>
<td>68*</td>
<td>100</td>
</tr>
<tr>
<td>10.0 $\mu$g B43-PAP it qwk x 4 wk</td>
<td>10</td>
<td>29</td>
<td>37</td>
</tr>
<tr>
<td>2.5 $\mu$g B43-PAP ip qwk x 4 wk</td>
<td>10</td>
<td>44</td>
<td>100</td>
</tr>
<tr>
<td>20 $\mu$g B43-PAP ip divided qd x 3d</td>
<td>10</td>
<td>65</td>
<td>100</td>
</tr>
<tr>
<td>15 $\mu$g methotrexate it qwk x 4 wk</td>
<td>10</td>
<td>49</td>
<td>100</td>
</tr>
</tbody>
</table>

SCID mice were injected IV with $1 \times 10^6$ NALM-6 cells. Administration of it B43-PAP or methotrexate was done weekly for 4 weeks, starting on day 14 after inoculation of leukemic cells. Controls were treated with normal saline (NS). Mice were monitored for paralysis and survival and data analysis was performed as described in Materials and Methods.

Abbreviations: it, intrathecal; ip, intraperitoneal; qwk, every week.

* $P < .0001.$

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**Fig 4. Efficacy of intrathecal B43-PAP immunotoxin therapy against xenografted human CNS leukemia in SCID mice.** SCID mice were injected IV with $1 \times 10^6$ NALM-6 cells. B43-PAP (2.5 $\mu$g per dose) or methotrexate (15 $\mu$g per dose) was administered intrathecally weekly for 4 weeks starting on day 14 after inoculation with leukemia cells. Controls received normal saline. The cumulative proportion of mice surviving event-free are shown according to the number of days after inoculation of leukemia cells.
BIOTHERAPY OF CNS LEUKEMIA

Fig 5. Absence of occult CNS leukemia after intrathecal (i.t.) B43-PAP immunotoxin therapy. (A, B) PCR detection of human DNA in BM and/or brain from normal saline (NS)-, i.t. methotrexate-, or intraperitoneal (i.p.) B43-PAP-treated SCID mouse controls. SCID mouse 4943 was treated with i.t. B43-PAP (2.5 μg per dose every week × 4 weeks). (C, D) BM and brain specimens from normal SCID mice (not inoculated with any human cells) were mixed with various amounts of NALM-6 DNA to determine the sensitivity of the PCR assay. Less than 0.001% human DNA contamination was observed in the brain or BM from two SCID mice (SCID mice 4943 and 4985) treated with B43-PAP (2.5 μg per dose every week × 4 weeks). SCID mouse 5053 was a control mouse treated with i.t. B43-PAP (2.5 μg per dose every week × 4 weeks) starting 1 week after inoculation with NALM-6 leukemia cells. Human DNA was observed in the BMs as well as brains of SCID mice treated with i.t. methotrexate (15 μg per dose every week with the intent of a 4-week therapy; SCID mice 4848 and 4847). Both mouse 4848 and mouse 4847 were electively killed on day 48 after becoming paralyzed secondary to CNS leukemia.

The hypothesis is supported by the fact that in the described SCID model, massive medullary and visceral leukemic burdens did not cause morbidity; neurologic signs of CNS disease were the first clinical evidence for onset of human leukemia.

There was no clinical or morphologic evidence of toxicity in mice given four weekly intrathecal treatments with 2.5 μg B43-PAP, a dose level that produced significantly prolonged survival and apparent cure in some SCID mice. However, intrathecal administration of B43-PAP at dose levels ≥10 μg per weekly single dose caused major damage to the spinal cord. Interestingly, neurotoxicity of intrathecal B43-PAP appeared to be cumulative. Only one mouse became paralyzed after a single intrathecal injection of 10 μg, whereas paralysis developed in the others only after multiple weekly injections of 10 μg. Damage only occurred near the site of injection, suggesting that neurotoxicity is caused by high local concentrations of B43-PAP, a situation that might be prevented by slow infusion of a dilute solution of the immunotoxin. This hypothesis can be tested in larger species.

The SCID mouse model of human CNS leukemia described in this report provides a basis for future comparative studies of the toxicity and efficacy of novel CNS therapy regimens in B-lineage ALL. These experiments provide a paradigm for the critical evaluation of a new CNS therapy regimen in B-lineage ALL. This report also extends previous studies that showed the potential therapeutic benefit of in-
Fig 6. Toxicity of intrathecal B43-PAP immunotoxin therapy. Caudal spinal cord of an SCID mouse 5 days after the third weekly intrathecal injection of 10 μg B43-PAP immunotoxin. There is massive necrosis of the parenchyma of the spinal cord, as well as vacuolization of ventral nerve roots. H&E stain; OM: (A), × 50; (B), × 100.

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Biotherapy for xenografted human central nervous system leukemia in mice with severe combined immunodeficiency using B43 (anti-CD19)- pokeweed antiviral protein immunotoxin

R Gunther, LM Chelstrom, L Tuel-Ahlgren, J Simon, DE Myers and FM Uckun