Two Serologic Markers to Monitor the Engraftment, Growth, and Treatment Response of Human Leukemias in Severe Combined Immunodeficient Mice

By Francisco Arguello, Judith A. Sterry, Yan Z. Zhao, Mark R.A. Alexander, Robert H. Shoemaker, and Harvey J. Cohen

We have investigated human lactate dehydrogenase (LDH) isoenzymes and human nuclear matrix protein 41/7 (NMP 41/7) as potential serologic markers to monitor the course of human leukemia in severe combined immunodeficient (SCID) mice. Following the transplanation of 10⁶ human acute lymphoblastic leukemia (ALL) Nalm-6 cells, human specific LDH isoenzymes were measurable in the serum of SCID mice as early as 7 days after transplantation, although serum total LDH increased in some animals as early as 5 days after transplantation. Human NMP 41/7 was measurable in all animals at day 15 after leukemia cell injection. Serum levels of total LDH, human specific LDH and NMP 41/7 increased progressively over time, reaching total LDH levels as high as 50,000 U/L at day 25 after transplantation. To determine whether the levels of LDH and NMP 41/7 in serum were a reflection of human tumor burden, we studied these serologic markers in SCID mice bearing measurable subcutaneous human neuroblastoma tumors, or compared the serum levels of these markers with the number of human leukemia CD10⁺ cells in the bone marrow of the SCID mice. The serum levels of total LDH, human specific LDH isoenzymes, and NMP 41/7 correlated well with tumor burden, and they drastically decreased or disappeared from serum after the human leukemia or neuroblastoma cells were selectively killed with a single intravenous (IV) injection of 1 to 3 μg diphtheria toxin (DT) (the cellular receptor for DT is present on human cells, but not on mouse cells). Paraplegic mice with central nervous system leukemia completely recovered after DT treatment. We conclude that measurements of serum levels of total LDH, human LDH isoenzymes, and NMP 41/7 are sensitive, quantitative, rapid, and easy to perform serologic methods useful to monitor the engraftment, progression, and treatment response of human leukemia in SCID mice.

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The discovery that human leukemia cells can be successfully grafted systemically into severe combined immunodeficient (SCID) mice, has created enormous interest in the use of SCID mice to study biological events or therapeutic strategies that can only be properly evaluated in vivo, eg, response to hematopoietic growth factors or cytokines, chemo-, radio-, immuno-, and gene-therapies. However, serious limitations exist in these animal models because human tumor engraftment, extension of the disease, and/or response to treatment are not amenable to direct observation. Thus, the presence of human leukemia cells can only be documented through semiquantitative and time-consuming methods, such as immunohistochemistry of the mouse organs, flow cytometric analysis of bone marrow or other tissues using human-specific-antigen labeling with monoclonal antibodies (MoAb), probing for the presence of human DNA sequences in the SCID mouse tissues, flow cytometric analysis of cellular DNA content or radiometric techniques. Moreover, animals must be killed and the experiments terminated to perform these assessments.

Lactate dehydrogenase (LDH) is an enzyme present in all human cells catalyzing the pH dependent interconversion of lactate and pyruvate. Characteristically, human LDH can be separated into five different isoenzymes (LDH1 through LDH5) based on their electrophoretic mobility. LDH is one of the enzyme systems that is preferentially produced and retained by cancer cells to maintain tumor growth. It is well known that increased serum levels of LDH is a common feature in humans and animals with malignant tumors, and in particular, in those with hematopoietic malignancies. It is believed that the increased levels of LDH result from the leakage of the enzyme from dying cancer cells that occur during the high turnover of the tumor. In 1977, Pesce et al made the observation that human LDH isoenzymes could be detected against a background of mouse LDH isoenzymes and showed that human larynx and bladder carcinoma cells implanted subcutaneously in nude mice released enzymes into the host's blood circulation, and that these proteins could be used as markers of chemotherapeutic response.

The nuclear matrix is a nonhistone proteinaceous nucleoskeleton structure having attachment sites for DNA loops during DNA replication. The nuclear matrix has been implicated in transcriptional regulation of gene expression and primary transcription processing (for review, see Vemuri et al). The nuclear matrix appears to be both cell type and species specific. It has been recently found that NMP is solubilized and released into the culture supernatant during the process of cell death or apoptosis. An immunoassay kit to measure human nuclear matrix protein (NMP) has been developed and commercialized. The immunoassay reacts with an antigen designated as NMP 41/7, which appears to represent fragments or the whole human specific nuclear matrix protein designated by some researchers as the nuclear mitotic apparatus protein, or NuMA, first described in 1980.

Diphtheria toxin (DT) is a potent inhibitor of protein synthesis by catalyzing the adenosine diphosphate (ADP)-ribosylation of cytoplasmic elongation factor-2, a critical molec-
ular component of the cellular biosynthetic machinery. The biocactivity of DT requires binding of the toxin to a specific cell surface receptor, followed by its internalization and translocation into the cytoplasm (for review, see Collier and London). Almost since its discovery 100 years ago, it became evident that in contrast to humans, mice have a natural resistance to DT. The differential susceptibility of human and murine cells is largely determined by either the lack of diptheria toxin-cell receptors on murine cells, or due to the inability of DT to translocate into the cytosol of murine cells. This phenomenon offers the unique opportunity of achieving complete killing of diptheria toxin-sensitive human cells in a mouse host.

We reasoned that LDH and/or NMP could be useful markers to monitor the dynamic growth of human leukemia in SCID mice, as they would be released into the circulation during the death of cells that occurs as part of the rapid turnover of leukemia cells. In addition, these markers could be used as indicators of tumor response to experimental therapy, because LDH and NMP 41/7 proteins would be released from the cytoplasm into the blood circulation during the process of necrosis or apoptosis of leukemia cells induced by anticancer agents. In this study, we have also evaluated the use of DT as a positive-control in the treatment of human leukemias xenografted into SCID mice.

MATERIALS AND METHODS

Animals. Female Balb/c-SCID and C57BL/6 mice, aged 8 to 10 weeks old were supplied by The Jackson Laboratory (Bar Harbor, ME). Animals were maintained under the guidelines established by the National Institutes of Health (NIH) and the University of Rochester.

Cell lines and injection into animals. As a prototype of childhood ALL, we used the human pre-B ALL cell line Nalm-6, which has previously been shown to consistently produce systemic disease after transplantation of the cells in SCID mice. As a prototype of a measurable solid tumor of childhood, we used the human neuroblastoma cell line LA-N-1. Human pre-B ALL Nalm-6 cells and LA-N-1 human neuroblastoma cells were grown in RPMI-1640 medium with 15% fetal calf serum using standard culture conditions. Cell viability was determined by trypan blue exclusion, and only cell suspensions with greater than 90% viability were used. Animals received Nalm-6 cells at a dose of 10⁷ cells per mouse in a total volume of 0.1 mL of medium without serum either intravenously (IV) or intraarterially as previously described in detail. LA-N-1 cells were injected subcutaneously at a dose of 2 × 10⁶ cells per mouse in a 0.2 mL of medium without serum.

Blood/serum collection from SCID mice. Blood (~200 μL) was collected into four to six nonheparinized borosilicate glass microhematocrit capillary tubes (Baxter, McGraw Park, IL) from each mouse by retro-orbital puncture. Capillary tubes were microcentrifuged at high speed and the serum collected using rubber bulbs for capillary tubes (Baxter). In our hands, bleeding of mice by snipping off the tip of the tail with scissors yielded hemolyzed blood samples due to the constant squeezing of the tail. These and other methods to obtain blood from mice are described in detail by Mitruka and Rawnsley. Samples contaminated with erythrocytes or visibly hemolyzed were not used for LDH measurements. Serum samples were stored at −70°C until assayed for total LDH activity, LDH electrophoresis, or NMP 41/7. Rapid freezing and thawing was used to avoid losing LDH activity.

Determination of total LDH, LDH isoenzymes, and human NMP 41/7 in serum. Total LDH activity was determined by reflectance spectrophotometry using Kodak Ektachem Clinical Chemistry Slides (Eastman Kodak, Rochester, NY). LDH isoenzymes were determined by electrophoresis on cellulose acetate using LD Flur kit (Helena Laboratories, Beaumont, TX). The bands were quantitatively scanned using a UV EDC-Densitometer (Helena Laboratories). Because the basal levels of LDH in mice are high, and to obtain a better resolution of the electrophoretic bands, we diluted the mouse serum in a 1:5 or a higher ratio in saline solution. The relative amount of human specific LDH was estimated according to the formula: human specific LDH = (percentage of LDH3 + LDH4 + LDH5) × (total LDH).

NMP 41/7 in serum was determined using the commercial enzyme-linked immunosorbent assay (ELISA) kit Apo-Pak (PerSeptive Diagnostics, Cambridge, MA). Apo-Pak is a colorimetric biotin-streptavidin coupled enzyme immunonassay reaction, quantitatively measured on a spectrophotometer-plate reader at 490 nm.

Flow cytometry analysis. The bone marrow (BM) of both femurs from each mouse was collected by flushing out the cells, after snipping off the epiphyses, using a 27-gauge needle and medium without serum. Cell suspensions from bone marrow were washed in phosphate-buffered saline (PBS) and stained with saturating amounts of MoAb 44C10 (antihuman CALLA/CD10). CD10 was selected to identify Nalm-6 cells, because it is a marker for ALL and found on the surface of the majority of pre-B ALL cells. The normal human electrophoretic pattern of LDH isoenzymes is shown in Fig IA.

Characterization of SCID mouse and human specific LDH isoenzyme patterns. The normal serum total LDH values and the electrophoretic pattern of its isoenzymes were studied in intact SCID mice, and compared with those in humans. The normal human electrophoretic pattern of LDH isoenzymes in serum is shown in Fig 1A. The average total LDH in intact SCID mice was 1,924 U/L (±185). In contrast to the five electrophoretic bands reported to be present in the
SEROLOGIC MARKERS IN HUMAN XENOGRAFTS

Fig 2. LDH isoenzyme patterns of a SCID mouse xenografted with human leukemia cells. (A) Serum before transplantation; (B) 5 days after injection of Nalm-6 cells; (C) after 10 days; (D) after 15 days; (E) after 20 days; (F) after 25 days; (G) 5 days after DT treatment.

plasma of normal mice, the LDH pattern in serum of SCID mice showed only three clearly distinguishable bands (Fig 1B), corresponding to mouse LDH1, LDH2, and LDH5. However, when SCID mouse cells were lysed, the five electrophoretic mouse LDH bands could be visualized (Fig 1C). When human and SCID mouse LDH isoenzymes are both present in serum, such as in the case of SCID mice xenografted with human tumor cells, human LDH1 and LDH2 overlapped with the LDH1 and LDH2 of the SCID mouse (Figs 1A and B). The third and most prominent isoenzyme of the SCID mouse (mouse LDH5), has an electrophoretic mobility that falls between the human LDH3 and LDH4 (Fig 1D). We termed it “LDH5M” in this study, as being the only mouse LDH isoenzyme that is maintained and visible in a human-SCID mouse chimera (Fig 1D), and as a means to differentiate it from the human LDH5 in our discussion. Thus, human LDH3, LDH4, and LDH5 could be separated and quantitated as the relative amount of human specific LDH from the serum of xenografted SCID mice. These measurements do not reflect the absolute amount of human LDH because LDH 1 and LDH2 fractions are not being considered.

Total LDH and isoenzymes in human ALL-bearing SCID mice. Following the transplantation of 10⁶ Nalm-6 cells into five SCID mice, the first specific indication of human tumor cell engraftment became evident as early as 10 days after injection, documented by the increase in LDH1 and LDH2 and the appearance of human LDH3 (Fig 2A through C). However, serum total LDH increased in some animals as early as 5 days after transplantation, and continued increasing progressively over time in all animals, to reach total LDH levels as high as 50,000 IU/L at day 25 after leukemia cell transplantation (Fig 3). Human specific LDH3 and LDH4 in SCID mice were identifiable and quantifiable in all animals at day 15 (Fig 2D), and human specific LDH5 became measurable at day 20 after transplantation (Fig 2E). Conversely, mouse-specific LDH5M decreased proportionally from 80% at day 0, to 8.8% of the total LDH at day 25. At day 25, some mice were losing weight and developed clinical evidence of CNS involvement, characterized by paraplegia of the hind limbs. Tumor-bearing mice were then treated IV with 1 μg DT. Six hours after DT treatment, the total LDH increased dramatically to values >60,000 IU/L (data not shown). Five days after DT treatment, the total LDH returned to basal, pretreatment levels (Fig 3), and human specific LDH4 and LDH5 disappeared from the serum (Fig 2G). LDH3 decreased significantly by that time (Fig 2G). Thus, the differential reduction of total or human specific LDH that occurred a few days after experimental treatment appeared to be a more accurate indicator of the tumor mass destroyed than the acute and transient increase of these proteins soon after treatment. Mice recovered completely after DT treatment and did not have any histologic evidence of tumor involvement at autopsy 10 days after treatment. Two non-DT treated mice died before reaching day 30 posttransplantation. At autopsy, the spleens of these mice were found to be enlarged, and histopathologic analysis demonstrated CNS involvement, complete replacement of the bone marrow, and growth of the leukemia cells in the soft tissue surrounding the long bones and spine. In the spine, leukemia cells invaded into the spinal canal through the

Fig 3. Effect of xenografted human leukemia cells on serum total LDH of SCID mice. Total LDH levels of five mice transplanted with 10⁶ human Nalm-6 cells. At day 25, mice 1, 2, and 4 were treated with 1 μg DT. M, mouse number.
bone foramina of the basivertebral vein. In the spinal cord, leukemia cells adopted a perivascular arrangement in the small vessels, a phenomenon also observed in human CNS leukemia.

**Human specific LDH and NMP 41/7 in xenografted SCID mice.** As indicated above, NMP 41/7 is solubilized and released into culture supernatant by dying cells. To determine whether solubilized NMP 41/7 could enter into the bloodstream from an extravascular compartment and whether it could be detected and quantitated in mouse serum, different doses of Nalm-6 cells lysed by repeated freezing and thawing were injected into the peritoneal cavity of intact C57BL/6 mice. NMP 41/7 was detected soon after and up to hours after intraperitoneal (IP) injection of as few as $10^6$ lysed human Nalm-6 cells per mouse (Fig 4). NMP 41/7 was undetected in intact C57BL/6 or SCID mice. Thus, the results demonstrated that human NMP 41/7 can enter the bloodstream from an extravascular compartment and can be detected and quantitated in the serum of mice.

To determine whether NMP 41/7 could be used in conjunction with, or as an alternative to, LDH in monitoring tumor burden, we injected five SCID mice with Nalm-6 cells, and assayed for LDH and NMP 41/7 at day 7, 15, 25, and 35 after tumor cell injection. At day 25, the animals were treated IV with 1 μg DT. The similarity of the results between human specific LDH and NMP was striking (Fig 5). Again, total LDH increased in a similar fashion to that described above and shown in Fig 3 and returned to basal levels 5 days after DT treatment. Also, the animals recovered completely after DT treatment. Clinical recurrence of the disease was observed in all animals about 25 days after DT treatment, and it was presaged by the increase in LDH and reappearance of human LDH isoenzymes in the serum (data not shown).
Table 1. Correlation of Serum Levels of Total LDH, Human Specific LDH, and NMP 41/7 With the Number of CD10+ Cells Found in the BM of SCID Mice at Different Time Points After Transplantation of NALM-6 Cells

<table>
<thead>
<tr>
<th>Days After Transplantation</th>
<th>CD10+ Cells* × 10^6 (±)</th>
<th>Total LDH (U/L)</th>
<th>Human Specific LDH (U/L)</th>
<th>NMP 41/7 (U/mL)</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>37.54 (4.5)</td>
<td>1,542±</td>
<td>0±</td>
<td>0±</td>
</tr>
<tr>
<td>8</td>
<td>66.2 (7.2)</td>
<td>2,182±</td>
<td>163±</td>
<td>0±</td>
</tr>
<tr>
<td>11</td>
<td>60.0 (7.4)</td>
<td>2,854±</td>
<td>256±</td>
<td>0±</td>
</tr>
<tr>
<td>16</td>
<td>110.1 (13.7)</td>
<td>7,972±</td>
<td>8,819±</td>
<td>456±</td>
</tr>
<tr>
<td>21</td>
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<td>13,352±</td>
<td>8,819±</td>
<td>1,765±</td>
</tr>
<tr>
<td>25</td>
<td>962.2 (59.4)</td>
<td>43,890±</td>
<td>26,794±</td>
<td>5,170±</td>
</tr>
</tbody>
</table>

Correlation coefficient with number of CD10+ cells

0.95 0.92 0.93

* Number of cells recovered from both femurs per mouse.
† Relative amount of human specific LDH, see Materials and Methods.
‡ Assayed obtained from two examined mice per time point.

was assayed for total LDH, LDH electrophoresis, and NMP 41/7 immunoassay. As shown in Table 1, the relative number of CD10+ cells correlated with total LDH, human specific LDH, and to a lesser degree, with NMP 41/7.

We also compared the levels in serum of LDH and NMP in animals bearing measurable subcutaneous tumor masses. Five SCID mice were transplanted subcutaneously with 2 × 10^6 LA-N-1 human neuroblastoma cells. Blood samples were obtained at day 30, 48, 54, 59, and 84 after transplantation. At day 54, the animals were treated IV with 3 µg of diphtheria toxin per mouse, except for two mice that were killed to document the growth of neuroblastoma tumor by histopathology. The tumor volume in each mouse and their respective levels of human specific LDH and NMP 41/7 are shown in Fig 6. Again, there was a parallel increase of these serologic markers with tumor growth in the animals. Subcutaneous tumors regressed completely about 2 months after DT treatment. The LDH isoenzyme pattern in a SCID mouse transplanted with the human neuroblastoma cells is shown in Fig 7 for comparison.

DISCUSSION

Despite highly effective chemotherapy for childhood leukemia, considerable interest and research continues to be directed to those patients who are not cured with modern treatments. In an attempt to better understand the biology of this disease and in an effort to develop better therapeutic strategies, the transplantation of normal and transformed human hematopoietic cells into SCID mice has become an important research tool in hematology.

In the present study, we evaluated LDH and NMP 41/7 in serum as potential methods to monitor the dynamic growth of human leukemias in SCID mice. A system like this would be particularly useful in studies in which the evolution of the disease is being assessed in response to experimental treatment with cytokines, chemo-, radio-, immuno- and gene-therapies.7-14 With a human pre-B ALL cell line, we found that simple measurements of total LDH in serum could provide reliable information on the degree of progression of the disease in the animals, as demonstrated by the corresponding increase in the number of CD10+ cells in the BM and/or the proportional increase in the concentration of human specific LDH isoenzymes. On the other hand, the selective ablation of human leukemia cells with DT resulted in a parallel decrease in total LDH and human specific LDH, as well as complete clinical recovery of the animals. This is perhaps not surprising, considering the fact that serum total LDH levels correlate well with disease activity and tumor

Fig 6. Correlation of human tumor burden with serum levels of human LDH and NMP 41/7. Five SCID mice were transplanted subcutaneously with a human neuroblastoma cell line to create a measurable tumor mass. At day 54, mice 1, 2, and 3 were treated with 3 µg DT. (A) Tumor volume; (B) relative amount of human specific LDH; (C) human NMP 41/7. Tumor in mouse no. 4 invaded into the peritoneal cavity. Thus, the tumor volume in the figure represents only the mass that was measurable subcutaneously. The specific LDH pattern of mouse no. 2 is shown in Fig 7. M, mouse number.
were lysed, the five LDH isoenzymes became demonstrable. There is the hypothetical possibility that severe tissue damage or hemolysis in the SCID mouse host (eg, secondary to cytotoxic therapy) may result not only in the increase of total LDH, but also in the showing of all mouse LDH isoenzymes, leaving human LDH 4 and 5 as the only human specific fractions to quantitate, because human LDH3 would overlap with mouse LDH3 and 4. Nonetheless, the presence of two human LDH fractions appear to be enough to reliably monitor human tumor xenografts.29 Concerning total LDH, the investigator has the option to deduct the total LDH found in nontumored-treated animals from the total LDH found in tumored-treated mice which, would give the relative human LDH fraction. NMP 41/7 is advantageous, as compared with LDH, in that its levels in serum are an absolute reflection of the cellular changes taking place in the human tumor xenograft because its measurement does not involve mouse proteins. Its disadvantage, is that it appears less sensitive, and the procedure is more laborious than LDH analyses.

On a purely theoretical basis, it appears reasonable to imply that these serologic markers could be applied to all human hematopoietic tumors xenografted into SCID mice, because the five characteristic human LDH isoenzymes and NMP 41/7 are universally distributed in human cells, regardless of their histology or lineage.19,29,33,36 We have observed these human markers, along with human immunoglobulins, in the serum of SCID mice reconstituted with normal human hematopoietic cells (peripheral blood leukocytes), as well as in nude mice grafted subcutaneously with the human promyelocytic leukemia cell line HL-60. We anticipate, however, that the amount of each isoenzyme fraction may vary from one type of leukemia to another because the LDH isoenzyme pattern is different among human hematopoietic cell lineages and/or stage of differentiation.18,55,56 Also, the amount of total LDH and NMP 41/7 released by the cells may vary from one type of leukemia to another because of the differences in the kinetics of cell growth and cell death among tumors.70,29 A reasonable suggestion is that individual investigators evaluate and choose the serologic marker that best fits the needs or objectives of his/her study. Researchers may also want to consider alternative procedures such as immunoassays for human specific LDH1 or LDH5 with MoAbs.

It has been previously shown that the most likely source of the increased LDH in humans and animals with cancer is the tumor itself, rather than damage of normal tissues or organ dysfunction.57 It is believed that the leakage of the enzyme occurs during the death of tumor cells in their constant and high turnover.26-28 However, indirect evidence suggests that the cells do not need to die to release this enzyme into the circulation. Hypoxia, electrolyte imbalances,26 reoxygenation after hypoxia,39 or calcium influx blockers can actually affect the rate and extent of enzyme leakage from tumors. Thus, tumor cell metabolism may also participate, at least in part, in the leakage of LDH from malignant tumor cells.

We have shown here that DT acts as a potent antileukemic agent due to the differential species-susceptibility to DT between humans and rodent cells. Despite being a protein,
DT was able to cross the blood-brain barrier and reversed paraplegia in mice with CNS leukemia. Similar findings have been observed with human brain tumors xenografted into the brain of nude rats. As a tool in leukemia research involving human xenografts, DT offers the opportunity to produce selective killing of human leukemia cells regardless of their lineage, and can provide a point of comparison to gauge new antileukemic agents. The use of DT may also contribute information about potential pharmacologic barriers, and could provide new insights concerning effective mechanisms to eradicate malignant cells with a defined molecular target.

The use of this serologic approach for future studies appears promising. We believe that this study represents an important first step to generate interest in this system. The analysis of LDH and its isoenzymes is performed routinely in clinical laboratories and could be rapidly implemented in most research laboratories. The immunoassay for NMP 41/7 is also easy to implement, requiring little more effort than LDH analysis. Thus, these methods should be evaluated in those studies in which biological and/or therapeutic approaches are being assessed after transplantation of freshly isolated or cultured leukemia and lymphoma cells in SCID mice to further characterize and validate this system. This system may prove to be a sensitive method to evaluate small or transient tumor responses to experimental treatment, which are otherwise undetectable by conventional methods.

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