Failure of Iron Chelators to Reduce Tumor Growth in Human Neuroblastoma Xenografts

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ABSTRACT

Neuroblastoma (NB) is a high risk tumor of childhood, and raised serum ferritin is an adverse prognostic factor. The hypothesis that iron chelation therapy impacts tumor status and patient prognosis through changes in iron metabolism has been systematically evaluated here in a xenograft model of human NB. One of two chelators was given in seven different regimens to nude mice xenografted s.c. with either IMR-32, an established cell line, or JBN-1, heterotransplanted directly from a patient.

Nude mice (a total of 160 in 24 cohorts) were given: desferrioxamine (DFO) by s.c. bolus or reservoir; 1,2-dimethyl-3-hydroxypyridin-4-one (DFO, L1, Lp, or orally; or saline. Measurements of mean Hb and liver iron levels were compared with corresponding saline controls per regimen as well as for pooled cohorts per agent for both cell lines. For IMR-32 xenografts, significant differences in Hb were achieved with L1 (10.9 g/dl pooled versus 13.7 g/dl controls) and in liver iron with DFO and L1 (235 µg/g and 306 µg/g, respectively, versus 520 µg/g). For JBN-1, the pattern was similar. With L1, Hb was 10.2 g/dl and controls were 11.7 g/dl (individual DFO cohorts were also significant); liver iron with DFO was 303 µg/g, liver iron with L1 was 270 µg/g, and controls were 387 µg/g. Additional therapy prior to tumor injection (67 mice and 10 cohorts) did not increase the depletion.

Despite documentation of iron depletion, no reductions in tumor engraftment, latency, or tumor size at end point were achieved in the chelator-treated mice, compared with controls populations. Accordingly, inclusion of these iron chelators in clinical trials for NB appears unwarranted.

INTRODUCTION

NB is one of the commonest solid tumors of childhood, and despite highly intensive treatment regimens, the prognosis for patients with advanced disease remains poor. Among the prognostic indicators that have been identified in patients with NB, an increased serum level at diagnosis of the iron storage protein ferritin is indicative of a significantly worse outcome (1). Ferritin has also been found in excessive quantities in the sera of patients with Hodgkin's disease (2), breast carcinomas (3), and hepatoblastomas (4), although the role of ferritin in malignancies remains unclear.

The origin of ferritin in patient's sera appears to be tumor derived, because the protein is found predominantly in its glycosylated form, indicating that it has been actively secreted (5). Furthermore, the ferritin associated with malignancies differs from the normal liver ferritin in the proportions of the two subunit types from which it is composed (6). Sera of nude mice bearing NB xenografts have been shown to contain human ferritin, further evidence that the protein is secreted by the tumor (7).

In an attempt to explain the role of ferritin in NB, it has been postulated that the protein may provide a growth advantage to NB cells (8). The most likely mechanism is preferential iron trapping by the ferritin-secreting tumor cells (9). It has been shown that there is a reproducible cytotoxic effect when NB cell lines are treated in vitro with the iron chelator DFO, whereas other embryonic tissue or embryonic tumors tested were not similarly affected (8). We have previously confirmed the dose-related cytotoxic effect of DFO on three NB cell lines in vitro (10).

In this study, we sought to evaluate the effect of two iron-chelating agents in nude mice, i.e., DFO, which can only be used parenterally, and L1, an experimental oral agent. The latter is one of a class of hydroxyxoyrindones that are effective iron chelators in vitro (11) and in vivo (12) and are presently under investigation for use in iron-overloaded patients (13). Initially, we determined the tolerable dose of each drug in our population of laboratory animals. By use of the iron-chelating agents, we proceeded to reduce the iron stores in cohorts of nude mice. Furthermore, we sought to evaluate the effect of iron chelation on the growth of human NB xenografts in the animal model.

MATERIALS AND METHODS

Neuroblastoma Cells

IMR-32 cells were obtained from the American Type Culture Collection (Rockville, MD). Cell lines grew as monolayers in RPMI 1640 (ICN Biochemicals, Sydney, Australia) supplemented with HEPES buffer (20 mM), L-glutamine (2 mM), 10% fetal calf serum, and 0.112% sodium bicarbonate, all purchased from ICN Biochemicals. Cells were tested and found to be free of Mycoplasma.

JBN-1 cells were derived in our laboratory by direct s.c. heterotransplantation and subsequent in vivo maintenance of clinical material from a child with advanced NB. The patient had increased serum ferritin at diagnosis (220 µg/l), and N-myc was amplified in the tumor cells. The latency time from the injection of cells until the appearance of tumor was constant, and serial histopathological assessment remained consistent with the tumor of origin.

Nude Mice and Procedures

Nude mice (nu/nu) of BALB/c background (bred by the Specific Pathogen Free Biological Facility, University of New South Wales, Sydney, NSW, Australia) were housed in a controlled environment in filtered cages, given sterile water, and fed ad libitum. Cages were autoclaved and changed weekly. Female mice 6–8 weeks of age and weighing 15–20 g were used. All procedures were carried out in a laminar flow hood. All experimental mice were observed for changes in condition and weighed daily.

 Procedures were carried out in accordance with permits issued by the University of New South Wales Animal Research Authority and under the guidelines of the National Health and Medical Research Council of Australia. Mice were killed by anesthetic overdose when they showed signs of poor health, persistent weight loss, when the tumours showed signs of perforation, or when experimental end points were reached.

Reservoir Installation. s.c. reservoirs (Alzet osmotic pump 2002; Alza Corp., Palo Alto, CA) were used to deliver some s.c. treatment. Mice allocated to have reservoirs inserted were anesthetized with inhaled enflurane (1% with air) and i.p. fentanyl (20% solution with 0.9% NaCl). Reservoirs were filled with DFO and installed through a 1-cm transverse incision in the flank. Reservoirs were changed every 2 weeks.

Received 9/25/97; accepted 11/26/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by the Children's Cancer Institute Australia and the Sydney Children's Hospital Foundation.

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2 The abbreviations used are: NB, neuroblastoma; DFO, desferrioxamine; L1, 1,2-dimethyl-3-hydroxypyridin-4-one; b.d., twice daily; MTD, maximum tolerated dose; Hb, hemoglobin.
Xenografting. For injection into nude mice, cultured IMR-32 cells were removed from tissue culture flasks with EDTA (1 mM). The cells were washed in RPMI 1640, and cell clumps were dissociated by gentle pipetting with a Pasteur pipette. Cell number was determined by counting on a hemocytometer, and viability was established by trypan blue exclusion. Cell suspensions of 10^6 cells/mL were prepared, and ice-cold attachment matrix (Matrigel; Integrated Sciences P/L, Sydney, Australia) was added (1 ml of cells/0.3 ml of Matrigel), immediately prior to injection. The cell suspension was injected s.c. through a 25-gauge needle (0.3 mL) into each flank of anesthetized nude mice (14). The in vivo passages were periodically renewed from cryopreserved tumor stocks.

The JBN-1 cell line was established and maintained as a s.c. tumor in nude mice. Tumors for passage were excised under sterile conditions, and cells were separated in RPMI 1640 and teased out with scalpel blades. A single cell suspension (10^7 cells/mL) was prepared by pipetting; the cells were counted, mixed with Matrigel, and re-injected using a 25-gauge needle into 6-8-week-old nude mice as described above.

To determine the effect of NB xenograft growth (+ saline treatment) on the iron status of nude mice, the Hb, serum ferritin, and the liver iron levels were measured 8 weeks after tumor cell injection in xenografted mice, which received either no treatment or a variety of saline regimens.

Iron Chelation

Mice were allocated to cohorts for treatment with iron chelator (Table 1). DFO (Desferal Ciba Geigy, Sydney, Australia) was dissolved in 0.9% NaCl solution to yield a concentrated solution (1000 mg/kg/dose), which was given i.p. and s.c. b.d. and by s.c. osmotic reservoir (357 mg/kg/day).

LI was a gift from Novartis (Basel, Switzerland). It was dissolved in 0.9% NaCl solution and administered (200 mg/kg/dose b.d.) i.p. (12) or s.c. (150 mg/kg/dose b.d.) and orally by intermittent orogastric tube (120 mg/kg/dose b.d.).

Determination of the MTD. The starting doses of iron chelators were determined by a review of the literature (11, 12). The MTD was defined as the dose level at which 30% or less of a cohort either died or lost weight in excess of 10%. Cohorts of 5-10 nude mice were used in toxicity experiments and continuing b.d. 5 days/week for 8 weeks. Tolerated doses of DFO were influenced by the method of administration and scheduling. The MTD of LI was determined after 10% reductions of dose in consecutive cohorts.

Treatment in Xenografted Nude Mice. To assess the effects of iron chelation therapy on NB xenografts, the first seven cohorts were treated with iron chelator from the day of tumor injection (Table 1). Treatment of xenografted animals commenced at the MTD for each route of administration. Treatments were given b.d., 5 days/week for 8 weeks. Tolerated doses of DFO were influenced by the method of administration and scheduling. The MTD of LI was determined after 10% reductions of dose in consecutive cohorts.

Monitoring. Animals were weighed daily, and tumor growth was monitored weekly by measurement with metal calipers of two perpendicular diameters. Tumor mass was calculated using the formula for ellipsoid volume: 

\[ V = \frac{1}{2} \times L \times W^2 \]  

where \( L \) is the longest axis, \( W \) is the shortest axis, and \( V \) is the volume. When two tumors were evaluated in one mouse, their volumes were added. Hb was checked prior to commencement of therapy, before injection of tumor cells, and at the end of treatment. Hb estimation was assayed by automated spectrophotometer (Sysmex NE-8000; Toa Medical Electronics Co. Ltd., Kobe, Japan) on whole blood or when sample volume was small (<250 μL) by 1:5 dilution. After the eighth week from the time of tumor injection, all mice were killed, and blood was taken for Hb as well as serum ferritin estimation.

Ferritin in mouse sera was quantified by radioimmunoassay (Coat-a-Count Ferritin IRMA kit; Diagnostic Products Corp., Los Angeles, CA). In this assay, ferritin in the sample was caught between monoclonal anti-ferritin antibodies immobilized on the inside surface of polystyrene tubes and radio-labeled polyclonal antiferritin tracer. Unbound 125I-labeled antiferritin antibody was removed by decanting the reaction mixture and washing the tube. The ferritin concentration was directly proportional to the radioactivity in the tube. The radioactivity was measured using a gamma scintillation counter. The ferritin concentration in the samples was obtained by interpolation from a curve constructed from the standards provided (16, 17).

After the completion of therapy, postmortem liver iron assays were conducted for comparison of cohorts. Open liver biopsy specimens were freeze-dried overnight, weighed, and halved into duplicate Mini-akto tubes (Alltech Associates, Sydney, Australia). Samples were dissolved in HNO3 (200 μL) and heated (80°C for 30 min) and mixed with iron-free distilled water (800 μL) prior to analysis of total iron by electrothermal atomic absorption spectrometry (Varian Spectra AA-30; Technon P/L, Sydney, Australia), using iron nitrate as a standard and bovine liver as a control (Standard Reference Material 1577b; National Institute of Science and Technology; Ref. 18).

Statistical analyses for determination of differences between cohorts were carried out according to Student’s t test.

RESULTS

Eight weeks growth of NB xenografts (+ saline treatment) in the nude mice was in itself found to significantly reduce their Hb and liver iron levels compared to unxenografted animals (Table 2). The serum ferritin was significantly elevated in the mice bearing JBN-1 xenografts but unchanged from control levels in the nude mice growing IMR-32 tumors (Table 2).

Preliminary experiments on unxenografted animals determined the MTD of iron chelator to be used. The starting dose of DFO (1000 mg/kg/dose) given as a single bolus i.p. was tolerated by all of the nude mice tested. However, when this dose was given i.p. twice daily for 8 weeks, only six of nine mice tolerated the dose, and three died.

The same dose of DFO (1000 mg/kg/dose) was tolerated better when administered s.c. and was determined to be the MTD, producing no deaths and, with 3 of 10 animals, exhibiting >10% weight loss. The

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Dosage</th>
<th>Preinjection</th>
<th>Postinjection</th>
<th>Weeks of treatment</th>
<th>No. of mice</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>JBN-1</td>
<td>LI p.o.</td>
<td>150 mg/kg/dose b.d.</td>
<td>0</td>
<td>8</td>
<td>6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LI p.o.</td>
<td>120 mg/kg/dose b.d.</td>
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<td>8</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LI p.o.</td>
<td>120 mg/kg/dose b.d.</td>
<td>12</td>
<td>8</td>
<td>10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFO s.c. bolus</td>
<td>1000 mg/kg/dose b.d.</td>
<td>0</td>
<td>8</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFO s.c. reservoir</td>
<td>357 mg/kg/day</td>
<td>0</td>
<td>8</td>
<td>6</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFO s.c. reservoir</td>
<td>357 mg/kg/day</td>
<td>0</td>
<td>8</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFO s.c.</td>
<td>150 mg/kg/dose b.d.</td>
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<td>8</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
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<td>DFO s.c.</td>
<td>150 mg/kg/dose b.d.</td>
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<td>8</td>
<td>7</td>
<td>8</td>
<td></td>
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<tr>
<td></td>
<td>DFO s.c.</td>
<td>120 mg/kg/dose b.d.</td>
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<td>8</td>
<td>10</td>
<td>5</td>
<td></td>
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<tr>
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<td>DFO s.c.</td>
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<td>3</td>
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<tr>
<td></td>
<td>DFO s.c.</td>
<td>357 mg/kg/day</td>
<td>0</td>
<td>8</td>
<td>9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>IMR-32</td>
<td>LI p.o.</td>
<td>150 mg/kg/dose b.d.</td>
<td>0</td>
<td>8</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LI p.o.</td>
<td>120 mg/kg/dose b.d.</td>
<td>4</td>
<td>8</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFO s.c.</td>
<td>1000 mg/kg/dose b.d.</td>
<td>0</td>
<td>8</td>
<td>15</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DFO s.c.</td>
<td>357 mg/kg/day</td>
<td>0</td>
<td>8</td>
<td>9</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Iron chelation regimes for cohorts of nude mice xenografted with human neuroblastoma

Fifty-five nude mice bearing flank tumors of either IMR-32 or JBN-1 NB cells were allocated to one of seven cohorts for 8 weeks of iron chelation therapy. Five cohorts (42 nude mice) received 4 or 12 weeks of additional iron chelation prior to cell injection. Sixty-three xenografted control mice were treated in a corresponding manner (12 cohorts) with equal volumes of saline.
was determined by radioimmunoassay, and liver biopsy specimens were analyzed in duplicate by atomic absorption spectrometry to assess the liver iron level. Statistical analyses were performed according to Student’s t test.

After 8 weeks of xenograft growth, the mice were tested for Hb, serum ferritin, and liver iron levels. Hb was determined by automated spectrophotometry of whole blood, serum ferritin determined by radioimmunoassay, and liver biopsy specimens were analyzed in duplicate by atomic absorption spectrometry to assess the liver iron level. Statistical analyses were performed according to Student’s t test. The heterotransplanted mice treated with iron chelators showed evidence of further reduced body iron status (Tables 3 and 4). Effective iron chelation was manifest in the anemia and/or reduced liver iron level displayed by all cohorts compared to xenografted saline-treated controls. LI produced significant reductions in Hb levels in five of six treatment groups. The exception, JBN-1 xenografted mice pretreated for 12 weeks with LI, showed a significantly reduced liver iron content documenting a reduction in body iron levels. DFO was less effective at producing a significant Hb drop than LI, with only two of six treatment groups developing anemia. The IMR-32 xenografted cohorts treated with DFO showed no significant reduction in Hb level. However, in all three cohorts, there was a significant reduction in liver iron level in response to iron chelation therapy. Similarly, JBN-1 xenografted mice treated with DFO all showed evidence of reduced body iron level either in a significantly lower Hb or liver iron content or both.

After treatment of the xenografted nude mice with iron chelators, the serum ferritin levels were undetectable compared with control animals (Table 2), where the values were within the limits of detection for the radioimmunoassay used. Ferritin protein determination by Western blot methodology was barely able to detect the presence of ferritin in the sera of untreated mice growing JBN-1, the higher ferritin-secreting tumor. The amount of serum ferritin in iron chelator-treated mice was below the detectable range for these analyses.

In eight of the nine cohorts of nude mice treated with DFO or LI where liver iron content was measured, the results displayed significant reductions compared with saline-treated controls. The exception was the cohort of JBN-1 xenografted mice treated by DFO s.c. from the time of injection of cells which, however, became severely anemic in response to therapy; three mice had final Hb levels ≤8 g/dl.

Overall, pretreatment of animals with chelator for an additional time of 4 or 12 weeks prior to injection of cells did not cause further significant reductions in iron status, compared with xenografted animals treated with chelators only from the time of tumor injection. In four of the five pretreated cohorts (DFO or LI), the Hb and liver iron levels were not statistically different from corresponding cohorts without additional pretreatment (Tables 3 and 4). Although there was convincing evidence of total body iron reduction by 8 weeks of treatment with the chelators, there was no concomitant reduction in the growth of NB xenografts (Table 5). Analysis of the pooled data for tumor mass after 8 weeks treatment with either DFO or LI found no significant reduction in the size of IMR-32 or JBN-1 xenografts. One cohort, the mice pretreated with LI for 4 weeks prior to injection, appeared to show a small decrease (P < 0.05) in IMR-32 xenograft size when compared with its parallel control group. However, when compared to the larger combined IMR-32 group of similarly treated saline controls, the reduction in tumor mass failed to acquire statistical significance.

In an attempt to maximize achievable iron chelation, one cohort of mice was treated with LI for a total of 5 months, 12 weeks prior to tumor injection and 8 weeks afterward. During the course of this experiment, three animals died suddenly, without preceding weight loss, after 8, 10, and 16 weeks of LI treatment, respectively. Four animals required LI dose reductions to 90 mg/kg/day b.d. following >10% weight loss after 10, 10, 11, and 15 weeks therapy. Only three of nine mice tolerated the starting dose of 120 mg/kg/day b.d. for the entire duration of the experiment. At the conclusion of the study, there was no significant change in size of xenograft tumors in response to protracted LI iron chelation (Table 5).

**DISCUSSION**

Increased serum levels of ferritin at diagnosis are associated with poor prognosis in patients with NB. Several strands of evidence suggest that the ferritin is tumor secreted. The proportion of ferritin H and L chains in serum of a patient with NB differs from that found in normal liver or spleen ferritin of the same patient. The glycosylated nature of the malignancy-associated ferritin supports it as a poor prognosis marker in NB. Several strands of evidence suggest that the ferritin is tumor secreted. The proportion of ferritin H and L chains in serum of a patient with NB differs from that found in normal liver or spleen ferritin of the same patient. The glycosylated nature of the malignancy-associated ferritin supports it as a poor prognosis marker in NB.

Table 3 Effect of iron chelators on IMR-32 xenografts in nude mice

<table>
<thead>
<tr>
<th>Xeno graft</th>
<th>Hb (g/dl)</th>
<th>Serum ferritin (ng/ml)</th>
<th>Liver iron level (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>14.9 ± 0.1</td>
<td>7.7 ± 2.1</td>
<td>660 ± 33</td>
</tr>
<tr>
<td>IMR-32</td>
<td>13.7 ± 0.3</td>
<td>6.8 ± 1.1</td>
<td>520 ± 46</td>
</tr>
<tr>
<td>JBN-1</td>
<td>11.7 ± 0.4</td>
<td>19.8 ± 5.8</td>
<td>387 ± 23</td>
</tr>
</tbody>
</table>

*Mean ± SE.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hb (g/dl)</th>
<th>Liver iron level (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.7 ± 0.3</td>
<td>520 ± 46</td>
</tr>
<tr>
<td>DFO s.c. bolus</td>
<td>13.2 ± 0.5</td>
<td>ND</td>
</tr>
<tr>
<td>DFO reservoir</td>
<td>12.7 ± 0.9</td>
<td>233 ± 8*</td>
</tr>
<tr>
<td>DFO reservoir +4 weeks pretreated</td>
<td>13.7 ± 0.4</td>
<td>240 ± 11*</td>
</tr>
<tr>
<td>DFO total</td>
<td>13.5 ± 0.5</td>
<td>235 ± 7*</td>
</tr>
<tr>
<td>L1 i.p.</td>
<td>11.1 ± 0.5</td>
<td>6 ND</td>
</tr>
<tr>
<td>L1 p.o.</td>
<td>10.4 ± 1.3</td>
<td>7 ND</td>
</tr>
<tr>
<td>L1 p.o. +4 weeks pretreated</td>
<td>11.4 ± 1.3</td>
<td>306 ± 17*</td>
</tr>
<tr>
<td>L1 total</td>
<td>10.9 ± 0.7</td>
<td>23 306 ± 17*</td>
</tr>
</tbody>
</table>

*Mean ± SE.

| Number of samples tested in duplicate.
| Number of animals sampled.

| Number of animals sampled.
| ND, not done.
Table 4 Effect of iron chelators on JBN-1 xenografted nude mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hb</th>
<th>Liver iron level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/dL*</td>
<td>µg/g*</td>
</tr>
<tr>
<td>Control</td>
<td>11.7 ± 0.4</td>
<td>387 ± 23</td>
</tr>
<tr>
<td>DFO s.c. bolus</td>
<td>9.4 ± 1.4a</td>
<td>348 ± 21</td>
</tr>
<tr>
<td>DFO s.c. reservoir</td>
<td>14.2 ± 0.1</td>
<td>303 ± 7p</td>
</tr>
<tr>
<td>DFO s.c. reservoir + 4 weeks</td>
<td>7.9 ± 0.4a</td>
<td>246 ± 26p</td>
</tr>
<tr>
<td>Pretreated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DFO total</td>
<td>10.7 ± 0.8</td>
<td>303 ± 14b</td>
</tr>
<tr>
<td>L1 p.o.</td>
<td>8.6 ± 0.7</td>
<td>326 ± 28b</td>
</tr>
<tr>
<td>L1 p.o. + 4 weeks pretreated</td>
<td>10.2 ± 0.4</td>
<td>320 ± 15b</td>
</tr>
<tr>
<td>L1 p.o. + 12 weeks pretreated</td>
<td>11.5 ± 0.6</td>
<td>362 ± 25b</td>
</tr>
<tr>
<td>L1 total</td>
<td>10.2 ± 0.4</td>
<td>270 ± 17b</td>
</tr>
</tbody>
</table>

* Mean ± SE.
† Number of animals sampled.
‡ Number of samples tested in duplicate.
* This cohort was the only one of five pretreated cohorts that showed significant additional reductions, compared with the corresponding 8-week treated cohorts.

Table 5 Effect of iron chelation on tumor mass

<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatment</th>
<th>Treated</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>JBN-1</td>
<td>DFO s.c.</td>
<td>3.298 ± 0.546 (6)*</td>
<td>3.051 ± 0.70 (6)</td>
</tr>
<tr>
<td></td>
<td>DFO s.c. reservoir</td>
<td>0.558 ± 0.254 (6)*</td>
<td>0.316 ± 0.094 (9)</td>
</tr>
<tr>
<td></td>
<td>DFO s.c. reservoir + 4 weeks pretreated</td>
<td>2.786 ± 0.700 (5)*</td>
<td>3.282 ± 0.531 (10)</td>
</tr>
<tr>
<td></td>
<td>DFO total</td>
<td>2.180 ± 0.413 (7)*</td>
<td>2.071 ± 0.343 (43)</td>
</tr>
<tr>
<td></td>
<td>L1 p.o.</td>
<td>2.484 ± 0.324 (5)*</td>
<td>2.323 ± 0.082 (4)</td>
</tr>
<tr>
<td></td>
<td>L1 p.o. + 4 weeks pretreated</td>
<td>3.135 ± 0.649 (5)*</td>
<td>3.282 ± 0.531 (10)</td>
</tr>
<tr>
<td></td>
<td>L1 p.o. + 12 weeks pretreated</td>
<td>1.421 ± 0.529 (6)*</td>
<td>0.868 ± 0.349 (4)</td>
</tr>
<tr>
<td></td>
<td>L1 total</td>
<td>2.284 ± 0.334 (16)*</td>
<td>2.071 ± 0.343 (43)</td>
</tr>
<tr>
<td></td>
<td>DFO s.c.</td>
<td>0.840 ± 0.264 (11)*</td>
<td>0.325 ± 0.097 (6)</td>
</tr>
<tr>
<td></td>
<td>DFO reservoir</td>
<td>0.233 ± 0.091 (5)*</td>
<td>0.281 ± 0.061 (9)</td>
</tr>
<tr>
<td></td>
<td>DFO reservoir pretreated 4 weeks</td>
<td>1.814 ± 0.430 (5)*</td>
<td>2.086 ± 0.429 (10)</td>
</tr>
<tr>
<td></td>
<td>DFO total</td>
<td>0.923 ± 0.207 (23)*</td>
<td>1.199 ± 0.189 (42)</td>
</tr>
<tr>
<td></td>
<td>L1 i.p.</td>
<td>1.140 ± 0.274 (5)*</td>
<td>1.418 ± 0.264 (5)</td>
</tr>
<tr>
<td></td>
<td>L1 p.o.</td>
<td>1.600 ± 0.347 (5)*</td>
<td>0.760 ± 0.174 (5)</td>
</tr>
<tr>
<td></td>
<td>L1 p.o. pretreated 4 weeks</td>
<td>0.993 ± 0.381 (5)*</td>
<td>2.085 ± 0.429 (10)</td>
</tr>
<tr>
<td></td>
<td>L1 total</td>
<td>1.265 ± 0.196 (16)*</td>
<td>1.199 ± 0.189 (42)</td>
</tr>
</tbody>
</table>

* Mean ± SE.
† Comparison with controls was not statistically significant, P > 0.05.
patient prognosis through changes in iron metabolism has been system-
tically tested here in an animal model of human NB. Two NB cell
lines were chosen for xenografting into nude mice: IMR-32, an
immortalized cell line derived from a child with unknown ferritin
level at diagnosis (34) and with a favorable response to the cytotoxic
effect of DFO in our previous studies (10); and JBN-1, derived from a
child with NB in our institution with serum ferritin of 220 μg/L at
diagnosis. A convenient latency period of 4 weeks from injection of
cells to appearance of tumor was another feature of each cell line. 
Taking into consideration the rapid clearance of DFO when given by
i.p. or s.c. bolus (13), it was decided to also administer DFO (357
g/kg/day) by osmotic reservoir (200 μL). Continuous s.c. infusion is
the regimen that best reproduces the clinical situation.
To address the possibility that DFO cytotoxicity is independent of
its iron chelation activity, a second chelator, L1, has been included in
these studies. The chemical and pharmacological properties of L1 are
different to DFO. L1 is a lipophilic compound that penetrates bio-
genetic membranes well and is absorbed through the gastrointestinal
lining, providing the option to administer orally (35). DFO, being
lipophobic and hydrophilic, is poorly absorbed through the gut and
can only be given parenterally. L1 is a bidentate chelator and requires
three molecules of chelator to every iron atom, whereas DFO is a
hexadentate chelator, binding iron 1:1. Preliminary assessments have
suggested that L1 was the stronger chelator, but more recent evidence
has indicated that DFO is actually more effective at chelating iron
than L1 (35). Because of the 3:1 stoichiometry of the L1/iron
complex, its stability is strongly dependent on the L1 concentration, which
must be maintained at high levels to be effective (35). Significant
amounts of incomplete 2:1 or 1:1 complexes may coexist with the 3:1
complex and are thought to generate toxic oxide radicals via the
Fenton reaction, which are thought to be responsible for the side
effects of L1 (36). The cytotoxic action of DFO has been attributed to
ribonucleotide reductase inhibition (23). A cytotoxic effect similar to
DFO has been demonstrated with L1 on NB cells (28), although the
mechanism of action may differ (12, 35, 37).

The toxicity of L1 has been shown to vary between animal species
and humans (35). L1 was found in these studies to be an effective iron
chelator in nude mice, given orally in divided doses. At doses greater
than 120 mg/kg/dose, it was found to be toxic, causing occasional
severe bone marrow depression, diarrhea, and sudden death. The use
of L1 in children with thalassemia major and chronic iron overload is
presently under investigation, and careful monitoring of the patients’
blood counts has been suggested (38) for the early diagnosis of the
bone marrow toxicity.

The results of our experiments indicate that 8 weeks of NB xe-
nograft growth produced relative iron depletion in the nude mice.
Significant further reductions in Hb and liver iron levels were seen
when xenografted nude mice treated with iron chelator for 8 weeks
were compared to xenografted controls. The serum ferritin levels
became undetectably low for the radioimmunoassay used. These
results were documented with DFO given i.p., s.c., and by s.c.
reservoir and with L1 given i.p., s.c., and orally. Liver iron level was
measured as a sensitive reflection of body iron status (39) and Hb as
an indirect indicator of reduced body iron. Pretreatment for 1–3
months prior to tumor cell injection did not further reduce the Hb or
liver iron levels. Importantly, nude mice treated with iron chelators
for 2–5 months duration showed no reduction in tumor engraftment
rate, latency, or tumor size at end point.

A possible explanation for the discrepancy between in vitro (8, 10,
and in vivo outcome could be the relative iron-deficient environ-
ument and absence of human transferrin in the tissue culture medium
(39). The addition of iron chelator is lethal in a dose-dependent
manner in the closed system, where no alternate source of iron is
available. In vivo, however, iron is mobilized from the macrophages
and hepatocytes, normally in an attempt to maintain the integrity of
essential functions (40). In this context, the NB tumor appears to
compete successfully for reduced body iron. It may not be possible to
deplete an organism of iron sufficiently to affect the growth of
malignant cells without causing other major toxicity.

A potential clinical benefit of in vitro cytotoxicity may be the use of
DFO as a purging agent to reduce the likelihood of minimal residual
disease in harvested bone marrow. Iron chelator has been added
to bone marrow in a closed system, where it has been shown to
be cytotoxic to NB cells, without adversely affecting hematopoetic cell
precursors (41).

Using each of two iron chelators, the experimental animals in these
studies were successfully depleted of iron. This strategy has, however,
been ineffective at causing tumor shrinkage in the treated animals. On
the basis of these data, clinical trials using iron chelators in NB appear
inappropriate. Toxicity, due to severe iron deficiency or associated
with the individual iron chelator, is predicted to precede tumor
response.

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