Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 5948–5952, August 1989
Medical Sciences

Fatal syndrome in mice engrafted with cells producing high levels of the leukemia inhibitory factor

(cachexia/bone formation/tissue calcification/pancreatitis)

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Contributed by D. Metcalf, April 25, 1989

ABSTRACT **Cells of the murine hemopoietic cell line FDC-P1 were multiply infected with a retroviral construct containing cDNA encoding the leukemia inhibitory factor (LIF) to produce cells secreting high levels of LIF. Injection of these cells to unirradiated or irradiated syngeneic DBA/2 mice resulted in animals engrafted with LIF-producing cells in the marrow, spleen, and lymph nodes and with elevated serum LIF levels. These mice developed within 12–70 days a fatal syndrome characterized by cachexia, excess new bone formation, calcification in heart and skeletal muscle, pancreatitis, thymus atrophy, and abnormalities in the adrenal cortex and ovarian corpora lutea. Injection of mice with control FDC-P1 cells led to comparable organ engraftment, but the mice developed none of these lesions. The observations suggest that LIF may be a potent cachexia-inducing agent and may have marked effects on osteoblasts and calcium metabolism.**

The leukemia inhibitory factor (LIF) was purified (1, 2) and a LIF cDNA was cloned (3) on the basis of its capacity to induce differentiation in and to suppress cells of the murine myeloid leukemic line M1. LIF has been reported to have proliferative effects on one hemopoietic cell line (4) but not on normal hemopoietic progenitor cells (5), although LIF receptors are present on cells of the monocyte–macrophage lineage (6). Evidence of broader actions of LIF has come from the observations that LIF can release calcium from bone tissue *in vitro* (7) and has a powerful *in vitro* action in suppressing differentiation in normal pluripotential embryonic stem cells (8).

In the present experiments, mice were engrafted with a hemopoietic cell line modified to produce high levels of LIF in an attempt to induce tissue changes that might provide further clues to the physiological functions of this molecule.

MATERIALS AND METHODS

Mice. Mice used were female 8- to 12-week-old DBA/2J mice and were raised in this Institute under specific pathogen-free conditions.

Construction and Selection of LIF Retrovirus. The 629-base-pair (bp) *Eco*RI–*Xba* I fragment of LIF cDNA from pLIFNK3 (9) was ligated into the *Eco*RI–*Xba* I site of pIC-20H (10). The LIF cDNA fragment was then excised as a *Sal* I–*Xho* I fragment and ligated into the *Xho* I site of pMPZen (11). Helper-free MPZen(LIF) virus-producing fibroblast lines were made by cotransfecting Ψ 2 cells (12) with pSV2-neo (13) and pMPZen(LIF) as described (11). The lines were screened initially for LIF production and then for their efficiency in infecting FDC-P1 cells (FD cells) (11).

Superinfection of FD Cells. The continuous hemopoietic cell line FD was obtained originally from T. M. Dexter (Paterson Institute, Manchester, U.K.). It is not leukemogenic and is dependent for proliferation *in vitro* on stimulation by granulocyte–macrophage colony-stimulating factor (GM-CSF) or multipotential colony-stimulating factor (Multi-CSF) (interleukin 3) (14). Cloned lines of FD cells producing high levels of LIF (FD/LIF cells) were generated by 14 sequential 2-day cocultivations with MPZen(LIF) virus-producing Ψ 2 cells and cloned sublines tested for their ability to produce LIF. The highest LIF-producing clone was estimated by Southern blotting to contain ≈16 copies of the retroviral insert per diploid genome and to produce 9.6×10^5 units of LIF per 10^6 cells per 24 hr. FD/LIF clones were tested for possible helper virus recombination with MPZen(LIF) by seven sequential 2-day cocultivations with equal numbers (10^6) of NIH 3T3 cells in the presence of GM-CSF and Multi-CSF. The NIH 3T3 cells were washed and allowed to proliferate in the absence of CSF for 6 days. The culture medium was changed and 24-hr-conditioned medium was produced and tested for LIF activity. In two independent assays, the NIH 3T3 culture medium tested negative for LIF activity.

Analysis of Viral RNA. Total RNA was isolated from guanidine thiocyanate lysates of cell lines and various mouse tissues (15), and RNA blot (Northern) analyses were performed (16). The LIF probe was the 570-bp insert from cDNA clone pLIFmut1 (3), labeled with [α-^{32}P]dATP by random priming.

Injection of Cells. DBA/2 mice were subjected to 6 Gy whole-body irradiation (γ-rays generated by Co^{60} at 0.39 Gy/min at 1.5 m) or left unirradiated and then injected intravenously with 1×10^6 FD cells (20 mice, 0 Gy; 40 mice, 6 Gy) or 1×10^6 cells of the highest LIF-producing FD/LIF subline (40 mice, 0 Gy; 40 mice, 6 Gy).

Observations. When moribund, mice were exsanguinated, and femoral marrow, spleen, mesenteric lymph node, and peritoneal cells were collected. Cultures were performed of 25,000 or 2500 cells from these tissues for 1 week in agar medium containing 400 units of GM-CSF plus 400 units of Multi-CSF. After colony counting, cultures were fixed with 2.5% glutaraldehyde and then stained with Luxol-Fast Blue/hematoxylin. From the morphology and composition of the stained colonies, the frequencies of clonogenic FD or FD/LIF cells and of the various normal hemopoietic progenitor cells were established.

LIF production by cell suspensions was determined by incubating 1×10^6 cells per ml for 24 hr in Dulbecco's modified Eagle's medium containing 5% fetal calf serum and 1000 units of GM-CSF to maintain the viability of FD or FD/LIF cells.

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Abbreviations: LIF, leukemia inhibitory factor; CSF, colony-stimulating factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; Multi-CSF, multipotential colony-stimulating factor; FD, FDC-P1 cells; FD/LIF, LIF-producing FD cells.

At autopsy, tissues were fixed in 10% Formol/saline and then blocked in paraffin, sectioned, and stained with hematoxylin and eosin or other stains.

LIF Assays. Serial 1:1 dilutions of 0.1 ml of material to be assayed for LIF content were added to 1 ml of agar medium cultures containing 300 M1 leukemic cells. After 7 days of incubation, the percentage of differentiated colonies was determined in each culture (5). LIF units were determined on the basis that 50 units/ml is the concentration inducing half of the M1 colonies to differentiate. Assays were standardized by the use of purified recombinant LIF preparations of known activity.

RESULTS

General Results. Commencing 10 days after the injection of FD/LIF cells, some irradiated recipient mice began to show weight loss, ruffled hair, and an irritable behavior. Within days of exhibiting these signs, such mice became moribund, and 90% of irradiated recipients developed this syndrome within 40 days of injection (Fig. 1). Unirradiated mice injected with FD/LIF cells developed a similar syndrome but with a delayed onset 30 days after injection and with 90% becoming moribund within 70 days. Irradiated or unirradiated mice injected with control FD cells remained apparently healthy during the 70-day observation period. All observations were made on moribund FD/LIF-injected mice, and on each occasion control mice injected with FD cells were killed for comparative analysis.

Transcription and Levels of LIF in Recipients of FD/LIF Cells. The injection of FD/LIF cells to DBA/2 mice achieved in both irradiated and unirradiated recipients a resident population of these cells in the bone marrow, spleen, and mesenteric node. The mean frequency of clonogenic FD/LIF cells in these tissues varied from 0.5% to 4.0% and was comparable with the frequency of clonogenic FD cells in the corresponding tissues of recipients injected with FD cells.

RNA samples extracted from various organs of moribund FD/LIF mice were analyzed for expression of the MPZen-(LIF) viral mRNA species [genomic, 2.66 kilobases (kb); subgenomic, 2.30 kb] to assess the transcription of LIF in these organs. All spleens (31 of 31) from FD/LIF mice tested positive for LIF viral mRNA, and all spleens from FD control mice tested negative (e.g., Fig. 2). A survey of tissues from a small group of FD/LIF mice showed expression of MPZen-(LIF) mRNA in the spleen, femur, lung, and mesenteric lymph node but not in the kidney, salivary gland, skeletal muscle, pancreas, or heart (data not shown).

LIF was not detectable in the serum of 23 irradiated or unirradiated recipients of FD cells, but in 14 irradiated recipients of FD/LIF cells, serum LIF levels were 1400 ± 1980 (±SD) units/ml and in 23 unirradiated recipients were 1140 ± 1490 (±SD) units/ml.

LIF was detected in medium conditioned by the following tissues from recipients of FD/LIF cells: the femur plus

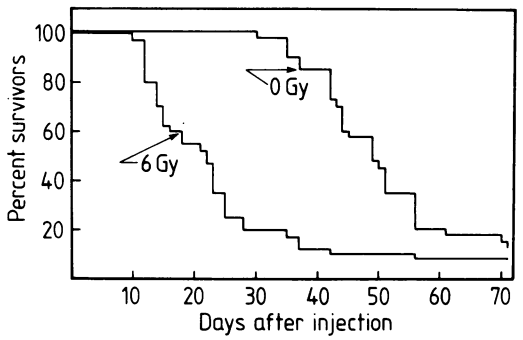

FIG. 1. Mortality in irradiated and unirradiated recipients of 1 × 10^6 FD/LIF cells.

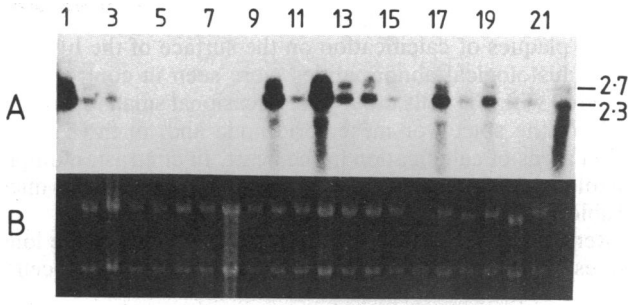

FIG. 2. Expression of MPZen(LIF) provirus in tissues from moribund mice injected with 10^6 FD or FD/LIF cells. Total RNA (2 μg) was isolated from spleens (lanes 2–15) and femurs (lanes 16–22) and subjected to RNA blot analysis. Lanes 2, 3, 10–15, and 17–22 contained RNA isolated from FD/LIF mice. Lanes 4–9 and 16 contained RNA from FD control mice. Lane 1 contained 0.2 μg of total RNA from FD/LIF cells. The positions of the genomic (2.7 kb) and subgenomic (2.3 kb) MPZen(LIF) transcripts are indicated. (*A*) Autoradiograph. (*B*) Ethidium bromide-stained gel.

marrow from 8 of 12 mice, the spleen from 15 of 21 mice, and the mesenteric node from 11 of 12 mice, levels ranging from 70 to 2400 units/ml. No LIF was detected in medium conditioned by cells from mice injected with FD cells.

Disease States in Recipients of FD/LIF Cells. Moribund recipients of FD/LIF cells exhibited obvious weight loss [irradiated recipients: FD cells (12 mice) 22.4 ± 1.8 g, FD/LIF cells (12 mice) 17.8 ± 2.1 g; unirradiated recipients: FD cells (16 mice) 24.2 ± 4.6 g, FD/LIF cells (24 mice) 17.9 ± 2.5 g] with loss of all subcutaneous and abdominal fat and generalized organ atrophy. Circulatory collapse was evident from the difficulty in bleeding the mice and the congested state of all organs after attempted exsanguination. Other visible abnormalities in FD/LIF mice were a soft, friable, pancreas of reduced size, marked thymus atrophy, moderate

Table 1. Frequency of tissue lesions in recipients of FD/LIF cells

Tissue lesion	Irradiated		Nonirradiated	
	FD	FD/LIF	FD	FD/LIF
Bone				
Excess osteoblasts	0/15	20/20	0/15	29/29
Excess bone formation	0/15	20/20	1/15	29/29
Bone resorption	0/15	14/20	0/15	24/29
Calcification				
Heart	2/16	14/20	1/13	14/24
Skeletal muscle	1/17	6/20	0/15	16/27
Liver				
Calcification	0/15	1/20	0/15	4/27
Hemopoiesis	0/15	18/20	0/15	24/27
Necrosis	0/15	5/20	0/15	17/27
Fibrosis	0/15	12/20	0/15	8/27
Pancreas				
Edema	0/13	15/19	2/15	27/28
Acinar degeneration	0/13	14/19	0/15	25/28
Infiltration	0/13	2/19	0/15	14/28
Adrenal cortex				
Brown degeneration	6/7	1/12	3/3	2/14
Ovary				
Follicles	4.0 ± 3.1	7.2 ± 5.2	11.3 ± 4.4	15.5 ± 6.0
Corpora lutea	9.6 ± 4.7	5.1 ± 4.8	10.5 ± 5.5	2.7 ± 2.8

Figures indicate the number of tissues with a particular abnormality versus the total surveyed. For the ovarian analysis, the figures represent the mean number of ovarian follicles or corpora lutea in the organ section ± SD in 17–41 ovaries from each of the various groups.

spleen enlargement, small adrenals of red color, and, in three mice, plaques of calcification on the surface of the liver.

No histological abnormalities were seen in control mice injected with FD cells other than occasional small foci of FD cells in the spleen or mesenteric node and, in three mice, small areas of calcification in the heart. In contrast, multiple histological abnormalities were observed in FD/LIF mice (Table 1, Fig. 3).

Remarkable changes were evident in the ends of the long bones and in the sternum of all recipients of FD/LIF cells. These included, even in unirradiated recipients, a depopulation of hemopoietic cells with a selective survival of granulocytic cells and the accumulation of elongated stellate cells, often oriented in parallel bundles. These stellate cells were enlarged close to bone surfaces and appeared to be osteoblasts engaged in new bone formation (Fig. 3*C*). Some mitotic activity was noted in these cells. There was irregularity in the width of the cortex and often widening of the foramina, leaving marrow cells in open contact with extraosseous tissue. Despite this evidence of bone resorption and occa-

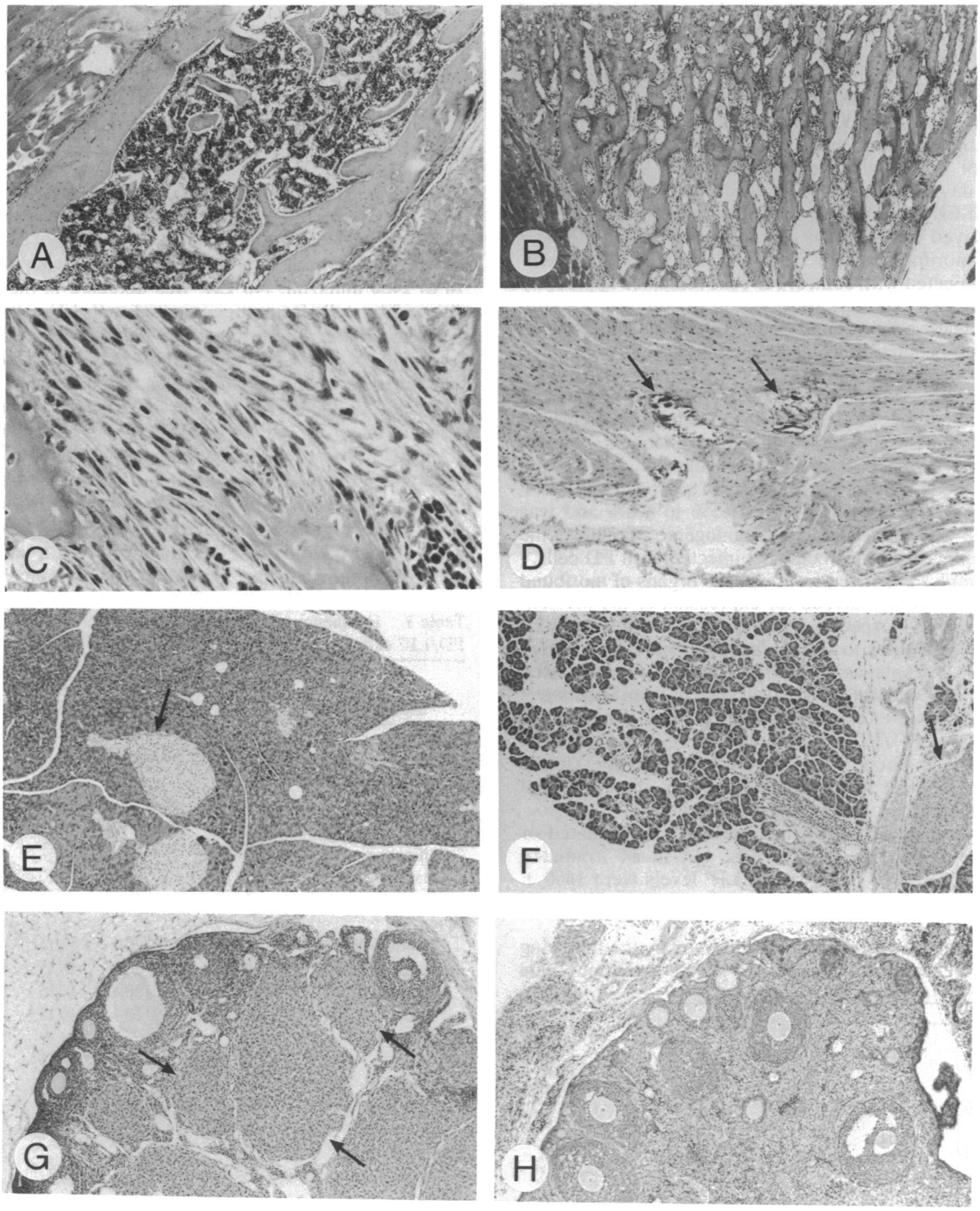

FIG. 3. (*A* and *B*) Femur from mouse injected with FD cells (*A*) and FD/LIF cells (*B*). Note the excess new bone formation and abnormal trabeculae in FD/LIF recipient. [Hematoxylin/eosin (H&E); ×54.] (*C*) Higher power view of marrow from a recipient of FD/LIF cells showing depletion of hemopoietic cells, excess numbers of osteoblasts, and new bone formation. (H&E; ×450.) (*D*) Heart of recipient of FD/LIF cells showing calcium deposits (arrows). (H&E; ×54.) (*E* and *F*) Pancreas from recipient of FD cells (*E*) and from recipient of FD/LIF cells (*F*). Note acinar degeneration and edema but normal islet (arrow). (H&E; ×54.) (*G* and *H*) Ovary from a recipient of FD cells (*G*) (the normal corpora lutea are indicated by arrows) and from a recipient of FD/LIF cells (*H*). Note the absence of corpora lutea in *H*. (H&E; ×54.)

sionally prominent osteoclasts, there was clear overall excess new bone formation, most evident as the formation of irregularly shaped trabeculae of bone occupying the marrow cavity (Fig. 3 *B* and *C*), an appearance resembling myelosclerosis. The excess new bone formation prevented attempts to insert a needle and flush marrow cells from the femur in 10 of 19 irradiated recipients and 11 of 19 unirradiated recipients of FD/LIF cells.

Focal areas of calcification were present in the myocardium of two-thirds of irradiated and unirradiated recipients of FD/LIF cells (Fig. 3*D*). Similar foci were present in skeletal muscle of many recipients but were less frequent in irradiated recipients (Table 1). Calcification appeared to initiate within the muscle cells and, unless the area was large, there was no associated cellular infiltration.

Liver parenchymal cells in recipients of FD/LIF cells were reduced in volume with dilation of the vessels. Most livers showed focal areas of extramedullary hemopoiesis and, particularly in unirradiated recipients, necrotic areas. Fibrosis around portal vessels was relatively common in irradiated recipients. A low percentage of the livers showed extensive foci of calcification, and such livers always showed necrotic areas.

The spleen was moderately enlarged with lymphoid follicle atrophy and excess hemopoiesis in the red pulp. The thymus was atrophic in all recipients of FD/LIF cells, with complete loss of cortical lymphocytes. The thymus cortex was normal in irradiated mice injected with FD cells. The pancreas showed dispersion of individual acini due to edema, with reduction in size and/or patchy necrosis of acinar cells and, in half of the unirradiated recipients, some infiltration mainly by mononuclear cells and granulocytes (Fig. 3*F*). The pancreatic islets were intact, and the mice showed no glucosuria.

The adrenals in mice injected with FD cells showed a prominent zone of lipid-containing cells in the inner cortex ("brown degeneration"), typical of DBA/2 mice. This zone was absent in most mice injected with FD/LIF cells, with reduction in organ size.

The ovaries of unirradiated, but not irradiated, recipients of FD/LIF cells were surrounded by an abnormal cellular capsule mainly containing lymphocytes. The frequency and maturation of ovarian follicles in FD/LIF recipients were not significantly altered, but there was a striking deficit of corpora lutea (Fig. 3*H*). Those that were present tended to be ill-defined with luteal cells of small volume.

Other than a reduction in cellular volume, no abnormalities were detected in recipients of FD/LIF cells in the lung, salivary gland, kidney, bladder, gut, mesenteric lymph node, brain, thyroid, or parathyroids.

DISCUSSION

After injection of FD cells into syngeneic DBA/2 mice, a progressively increasing population of these cells accumulates in the marrow, spleen, and lymphoid tissues (U. Dührsen and D.M., unpublished data). FD/LIF cells exhibited the same behavior and, as in previous studies using FD cells, the cellular accumulation was accelerated by preirradiating the recipients (17). Analyses for LIF mRNA and bioassays for LIF indicated that active production of LIF occurred in the marrow, spleen, and mesenteric node of recipients of FD/LIF cells, and there was a substantial elevation of circulating LIF levels. No elevation of LIF production or serum LIF levels was seen in control mice injected with FD cells.

In mice containing FD/LIF cells, a fatal cachexia developed within an interval as short as 12 days. The mice exhibited a novel syndrome of bone overgrowth with associated extramedullary hemopoiesis in the spleen and liver, calcification in muscle tissue and liver, pancreatitis, thymus atrophy, and adrenal and ovarian changes.

None of these abnormalities developed in irradiated or unirradiated recipients of FD cells, suggesting that the abnormalities were the consequence of excess LIF levels. The changes in the bone and bone marrow may have been associated with local production of LIF by resident FD/LIF cells. However, other organs such as the pancreas, muscle, and ovaries contained no obvious FD/LIF cells and, if the lesions were directly initiated by LIF, the LIF must have reached these tissues via the circulation.

The observed tissue damage might either have been the consequence of direct actions of LIF on these various cells or have been mediated indirectly by some LIF-initiated product. Osteoblasts exhibit LIF receptors, and the pancreas can bind significant amounts of injected ^{125}I-labeled LIF (D. J. Hilton, N. A. Nicola, and D.M., unpublished data). Potentially, therefore, the accumulation of osteoblasts with excess new bone formation and the pancreatic damage may have been direct effects of excess LIF stimulation.

The only agent previously known to induce cachexia is tumor necrosis factor (18). LIF may be another such agent or may merely induce the production of tumor necrosis factor. Many of the changes such as reduction in organ parenchymal volume, thymus cortex atrophy, loss of adrenal lipid, and defective corpora lutea formation may merely be secondary consequences of the cachectic state. The early death of some irradiated recipients of FD/LIF cells might be the combined consequence of irradiation-induced hemopoietic aplasia and organ damage plus early toxic effects of LIF.

The most consistent tissue abnormalities were an increased number of osteoblasts in the bone marrow with excess new bone formation, often in animals with increased bone resorption and calcium deposition in muscle tissue. Previous *in vitro* studies showed an ability of LIF to induce calcium release from bone tissue *in vitro* (7). The combination of new bone formation, bone resorption, and calcium deposition in tissues suggests a novel perturbation of calcium metabolism.

The experiments suggest that LIF, in excess concentrations, may be a highly toxic agent but that LIF may play a normal role in regulating bone formation and calcium metabolism. The latter findings suggest that LIF may have a clinical application in promoting new bone formation and calcification.

In the present animals engrafted with LIF-producing cells, the abnormalities induced offer unusual models for investigating disease states such as osteosclerosis, pancreatitis, and cachexia. The engraftment model has the potential to be extended to study the long-term effects of excess levels of any factor able to be produced by FD cells after retroviral insertion of the appropriate cDNA.

We are indebted to A. Futter, Y. Pattison, M. Lahn, and A. Wilson for careful technical assistance throughout this work; to S. Mihajlovic and V. Likiardopoulos for preparing the histological material; to I. Hariharan and S. Cory for the pMPZen construct; to K. Klingler for assistance in screening for helper virus contamination; and to V. Duhrsen, G. R. Johnson, and N. Gough for technical advice and gifts of reagents. This work was supported by the Carden Fellowship Fund of the Anti-Cancer Council of Victoria, The National Health and Medical Research Council, Canberra, and the AMRAD Corporation, Melbourne.

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