Production of 1,25-Dihydroxyvitamin D_3 by Human T Cell Lymphotrophic Virus-I-transformed Lymphocytes

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Abstract

The human T cell lymphotrophic virus type I (HTLV-I) has recently been identified in a T cell lymphoma associated with hypercalcemia and increased bone turnover. Since increased serum concentrations of 1,25-dihydroxyvitamin D have been reported in this disease, we have examined the capacity of HTLV-I-infected cord blood lymphocytes to metabolize 25-hydroxyvitamin D_3. Our results demonstrate that HTLV-I-infected cells have the capacity to metabolize 25-hydroxyvitamin D_3 to a substance that co-migrates with 1,25-dihydroxyvitamin D_3 by high performance liquid chromatography over a silica column using either 12% isopropanol in hexane or 5% isopropanol in dichloromethane. The metabolite binds to the 1,25-dihydroxyvitamin D_3 receptor in rat osteosarcoma cells and stimulates bone resorption in cultures of fetal rat long bones. Mass spectrometric analysis of the metabolite confirmed the presence of 1,25-dihydroxyvitamin D_3. Production of 1,25-dihydroxyvitamin D by lymphoma cells may contribute to the pathogenesis of the hypercalcemia seen in patients with HTLV-I-associated T cell lymphomas.

Introduction

The human T cell lymphotrophic virus type I (HTLV-I)[1] is a type C retrovirus that has recently been associated with a peculiar form of T cell lymphoma described in patients in the United States, Japan, and the West Indies (1). This tumor is characterized clinically by lymphocytosis, hepatosplenomegaly, and a particularly aggressive clinical course (1). Since hypercalcemia is an infrequent complication of most malignancies, it is remarkable that almost all of the patients studied to date with HTLV-I-

associated T cell lymphomas have developed a syndrome of increased bone turnover and hypercalcemia at some time during the course of their disease. Bone biopsies showing increased osteoclast activity in the absence of tumor invasion, and the induction of bone resorption by conditioned media harvested from tumor cells in vitro, strongly suggest that the hypercalcemia seen in this disorder is mediated by a humoral mechanism (2). The finding of increased circulating concentrations of 1,25-dihydroxyvitamin D [1,25(OH)$_2$D] in a patient with HTLV-I-positive lymphoma has raised the possibility that production of 1,25(OH)$_2$D by tumor lymphocytes may play a role in the pathogenesis of the hypercalcemia seen in this disorder (3). Accordingly, the purpose of this study was to examine the possibility that HTLV-I-transformed T lymphocytes have the capacity to convert 25-hydroxyvitamin D_3 [25(OH)D_3] to 1,25(OH)$_2D_3$. Human cord blood T lymphocytes immortalized by the HTLV-I virus after co-cultivation with lymphoma cells carrying this virus have been used as a model to study the characteristics of HTLV-I-induced neoplastic transformation (4, 5). These transformed cord blood cells express HTLV-I proteins and have identical morphologic and functional properties to the neoplastic T cells (4, 5). They have been shown to contain lobulated nuclei, to retain the potential for indefinite growth, and to demonstrate surface receptor characteristics and lymphokine production that match those of the HTLV-I-infected lymphoma cells (5).

Methods

Cells. The cord blood lymphocytes infected with HTLV-I used in these studies were the C91/PL cell line. The cells were analyzed for expression of HTLV antigens, extracellular virus, karyotype, and HLA profiles. The cells were shown to assume the morphologic and functional characteristics of the parent lymphoma cells and have been in continuous culture for several years (4, 5).

Metabolite production. The capacity of the virus-infected transformed cord blood lymphocytes to metabolize 25(OH)D_3 was determined in vitro. The cells were grown to a density of $\sim 10^6$ cells/ml in 10 ml of RPMI 1640 medium (KC Biological, Inc., Lenexa, KA) supplemented with 10% fetal bovine serum. The culture medium was replaced with serum-free RPMI 1640 medium 48 h before each assay. The cells were then incubated with $\sim 200,000$ dpm (1 pmol) ^{3}H-25(OH)D_3 (25-hydroxy-26,27-methyl-^{3}H-cholecalciferol, 170 Ci/mmol, Amersham Corp., Arlington Heights, IL) for a period of 24 h. At the end of this incubation the cells and medium were harvested, and the 25(OH)D_3 metabolites were extracted into 5 ml of acetonitrile. After the addition of 5 ml K$_2$HPO$_4$ buffer (0.4 M, pH 10.6), the extract was applied directly to a prewashed C18 Sep Pak cartridge (Waters Assoc., Millipore Corp., Milford, MA). The cartridge was then washed with 5 ml of distilled water followed by

3 ml of methanol/water (70:30). The 25(OH)D$_3$ metabolites, which remain adsorbed onto the C18 Sep Pak, were then eluted with 5 ml of acetonitrile (6). The 25(OH)D$_3$ metabolites were separated and purified by high performance liquid chromatography (HPLC) over a silica column (Zorbax Sil, DuPont) using either 12% isopropanol in hexane at a flow rate of 2 ml per minute or 5% isopropanol in dichloromethane at a flow rate of 1 ml per minute.

In order to examine the biochemical characteristics and molecular structure of this metabolite, HTLV-I transformed cord blood lymphocytes were incubated with serum-free medium containing approximately 1 μg of highly purified unlabelled 25(OH)D$_3$ (0.25 μM) for a period of 24 hours under the conditions described above. The 25(OH)D$_3$ metabolites were extracted and purified by HPLC over a silica column using 12% isopropanol in hexane as described above. Those fractions that eluted at the same position as ^{3}H-1,25(OH)$_2$D$_3$ were pooled and rechromatographed using the same solvent system. Those fractions that again eluted at the same position as ^{3}H-1,25(OH)$_2$D$_3$ were pooled, dried under nitrogen, and reconstituted for further characterization, which would include the capacity for binding to 1,25(OH)$_2$D receptors, stimulation of bone resorption, and mass spectrometry.

Receptor assay. Binding of the purified 25(OH)D$_3$ metabolite to the intracellular 1,25(OH)$_2$D receptor was determined using rat osteosarcoma cells as previously described (7).

Bone resorption assay. The capacity of the purified 25(OH)D$_3$ metabolite to stimulate bone resorption in vitro was examined using a bioassay of fetal rat long bones in organ culture (8), in the presence or absence of the 25(OH)D$_3$ metabolite, for a period of 48 h. Results are expressed as the ratio of the percent of the total ^{45}Ca released in the test groups compared to the percent of the total ^{45}Ca released in the control group. Differences between control and test groups were analyzed using the Student's t test for nonpaired samples.

Mass spectrometry. The HTLV-I metabolite produced by incubating 25(OH)D$_3$ (1 μg) with HTLV-I–transformed cord blood lymphocytes was purified by two consecutive HPLC runs using 12% isopropanol in hexane. ^{3}H-1,25(OH)$_2$D$_3$ (10,000 dpm or 0.1 pmol) was added to each sample as an internal marker before purification by HPLC. Approximately 50 ng of 1,25(OH)$_2$D$_3$ or the purified vitamin D$_3$ metabolite were placed in separate reaction vials with 20 μl N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA; Pierce Chemical Co., Rockford, IL) and heated at 60°C for 10 min. Gas chromatographic separation of the trimethylsilyl ether derivatives was accomplished with a 15-m × 0.32-mm i.d. BP-1 fused silica column (Scientific Glass Engineering Inc., Austin, TX) heated from 270°C to 280°C at 5°C/min after injection. Samples (10 μl) were introduced by means of a glass falling needle injector (R. H. Allen Co.) at an injection port temperature of 250°C. The head pressure of the helium carrier gas was 4 psi and the exit flow from the injector was 2 ml/min. The effluent from the gas chromatograph was admitted directly into the mass spectrometer through a heated transfer line, which was maintained at 250°C. Positive ion electron impact (70 eV) mass spectrometry was performed on a Finnigan-MAT 212 mass spectrometer in combination with an INCOS 2200 data system. The ion source temperature was 250°C for the gas chromatography mass spectrometric analyses and 150°C for the direct insertion probe studies. The accelerating voltage was 3 kV.

Results

The elution pattern of the 25(OH)D$_3$ metabolite produced by incubation of ^{3}H-25(OH)D$_3$ with HTLV-I–transformed cord blood lymphocytes revealed a peak of radioactivity that eluted with the same retention time as 1,25(OH)$_2$D$_3$ by HPLC over a silica column using 12% isopropanol in hexane (Fig. 1). In contrast, no conversion of ^{3}H-25(OH)D$_3$ to this more polar metabolite was produced by normal cord blood lymphocytes activated by incubation with 1% phytohemagglutinin for 48 h (data not shown). It has been reported that several 25(OH)D$_3$ metabolites
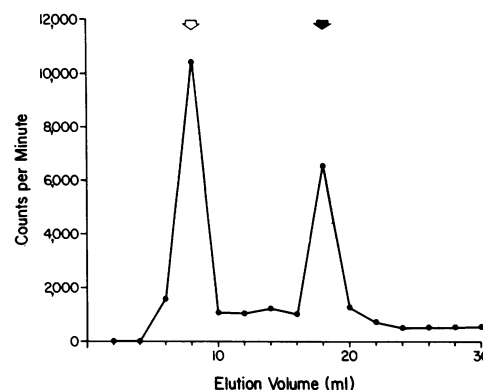

Figure 1. Elution pattern of the ^{3}H-vitamin D$_3$ metabolite produced by incubation of ^{3}H-25(OH)D$_3$ with HTLV-I–transformed cord blood lymphocytes. The vitamin D$_3$ metabolites were extracted and chromatographed over a Zorbax silica column (4.6 mm × 25 cm) using 12% isopropanol in hexane at a flow rate of 2 ml/min. The elution volumes of ^{3}H-25(OH)D$_3$ and ^{3}H-1,25(OH)$_2$D$_3$ are shown by the open and closed arrows, respectively.

co-elute with 1,25(OH)$_2$D$_3$ using a single solvent system of isopropanol in hexane (9, 10). Accordingly, those fractions containing the metabolite produced by HTLV-I cells that eluted with the same retention time as 1,25(OH)$_2$D$_3$ using 12% isopropanol in hexane were pooled and rechromatographed using 5% isopropanol in dichloromethane, a system that can separate 19-nor-10-keto 25(OH)D$_3$ from 1,25(OH)$_2$D$_3$ (9). The resulting elution profile was identical to that of 1,25(OH)$_2$D$_3$ (Fig. 2). The 25(OH)D$_3$ metabolite also eluted with the same retention time as 1,25(OH)$_2$D$_3$ by HPLC over an Ultrasphere ODS column using 22% water in methanol (data not shown). These results show that the 25(OH)D$_3$ metabolite produced by incubating ^{3}H-25(OH)D$_3$ with HTLV-transformed cord blood lymphocytes has the same chromatographic properties as 1,25(OH)$_2$D$_3$.

The 25(OH)D$_3$ metabolite under investigation also demonstrated biological properties characteristic of 1,25(OH)$_2$D$_3$. The purified 25(OH)D$_3$ metabolite was able to displace ^{3}H-1,25(OH)$_2$D$_3$ from intracellular receptors in rat osteosarcoma cells in a dose-dependent manner (Fig. 3). Moreover, when the
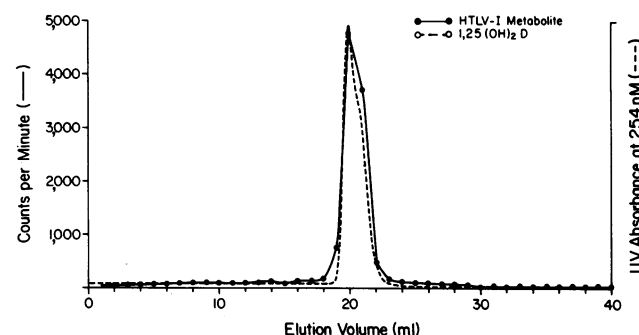

Figure 2. Elution pattern of the ^{3}H-vitamin D$_3$ metabolite produced by incubating ^{3}H-25(OH)D$_3$ with HTLV-I–transformed cord blood lymphocytes (solid line) compared with that of 1,25(OH)$_2$D$_3$ (broken line). Fraction 9 (elution volume 17–18 ml), which co-eluted with 1,25(OH)$_2$D$_3$ from a silica column using 12% isopropanol in hexane (Fig. 1), was rechromatographed on a silica column using 5% isopropanol in dichloromethane at a flow rate of 1 ml/min. The elution pattern of unlabeled 1,25(OH)$_2$D$_3$ was determined simultaneously by ultraviolet absorbance at 254 nm.
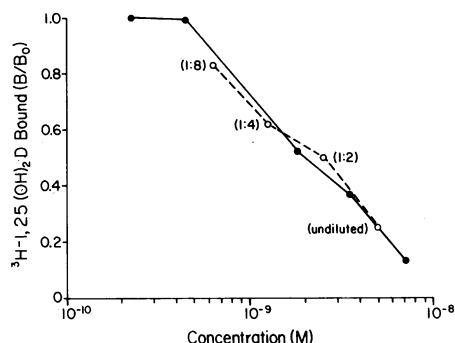
Figure 3. Displacement of ^{3}H-1,25(OH)$_2$D$_3$ from the intracellular 1,25(OH)$_2$D receptor prepared from rat osteosarcoma cells by 1,25(OH)$_2$D$_3$ (closed circles) or the vitamin D$_3$ metabolite produced by incubation of HTLV-I-transformed cord blood lymphocytes with 25(OH)D$_3$ for 24 h (open circles). The vitamin D$_3$ metabolite was purified by two sequential HPLC elutions over a silica column using 12% isopropanol in hexane. The dilutions of the vitamin D$_3$ metabolite are shown in parentheses. Results are expressed as a fraction of maximal specific binding of ^{3}H-1,25(OH)$_2$D$_3$ determined in the absence of competitor.

Figure 4. Electron impact (70 eV) mass spectra of the tris (trimethylsilyl) derivative of 1,25(OH)$_2$D$_3$ (top) and metabolite (bottom). Samples were introduced into the mass spectrometer in a quartz tube by means of a direct insertion probe. The ion source temperature was 150°C. Volatilization occurred at ~150°C.

purified 25(OH)D$_3$ metabolite was incubated in a bone resorption assay system, there was a significant increase in the amount of ^{45}Ca released in bones exposed to media containing the 25(OH)D$_3$ metabolite compared with those bones exposed to control media alone (Table I). Similar effects on bone resorption were produced by 1,25(OH)$_2$D$_3$ at concentrations of 10^{-8} M.

While it seemed unlikely that another 25(OH)D$_3$ metabolite could exhibit the same chromatographic and biological characteristics as 1,25(OH)$_2$D$_3$, identification of this metabolite remained speculative until its molecular structure was confirmed. Mass spectrometric analysis of the 25(OH)D$_3$ metabolite that eluted with the same retention time as 1,25(OH)$_2$D$_3$ over two HPLC runs using 12% isopropanol in hexane was performed. Fig. 4 shows the mass spectra obtained from the trimethylsilyl derivatives of standard 1,25(OH)$_2$D$_3$ (Fig. 4, top) and the metabolite (Fig. 4, bottom) after introduction by means of a direct insertion probe. Excellent agreement was seen not only between the spectra shown in Fig. 4 but also with previously published results (11). Both the standard and the metabolite volatilized at ~150°C and exhibited the following characteristic ions: a molecular ion at m/z 632; m/z 617, [M-CH$_3$]$^+$; m/z 542, [M-(CH$_3$)$_3$SiOH]$^+$; m/z 206, which indicates that two hydroxyl groups are present on the "A" ring; and m/z 131, which reflects hydroxylation at the 25-position. Further evaluation of the chemical nature of the metabolite was obtained by gas chromatography–mass spectrometry. It has previously been shown that 1,25(OH)$_2$D$_3$ can thermally rearrange to form two sterol isomers (12). These isomers, called the pyro and isopyro forms, are seen as two distinct gas chromatographic peaks. Both the standard and the metabolite behaved in this manner under the conditions of the present study, yielding peaks with retention times of 5.0 min (pyro) and 5.8 min (isopyro). The spectra for the major isomer (pyro) are shown in Fig. 5. Again the spectrum from the metabolite closely matched that of the standard. Thus, the identity of the metabolite as 1,25(OH)$_2$D$_3$ is further substantiated by the agreement with the thermal reactivity and gas chromatographic behavior of the standard and the mass spectra of the trimethylsilyl derivatives.

Table I. Bone-resorbing Activity of the Vitamin D$_3$ Metabolite*

Agent	Percent release of ^{45}Ca	Bone-resorbing activity (treated/control ratios of ^{45}Ca release)
Control	16.1±0.7	—
Metabolite	29.3±3.5	1.82±0.22‡
1,25(OH)$_2$D$_3$ (10^{-8} M)	30.2±3.2	1.88±0.20‡
(10^{-7} M)	42.5±4.6	2.64±0.29‡

* Purified by two consecutive HPLC elutions over a silica column using 12% isopropanol in hexane. Results are expressed as the percent ^{45}Ca released, with any treated/control ratio of >1.0 indicating stimulation of bone resorption. Statistical comparisons for bone-resorbing activity were made using Student's t test ($n = 4$).
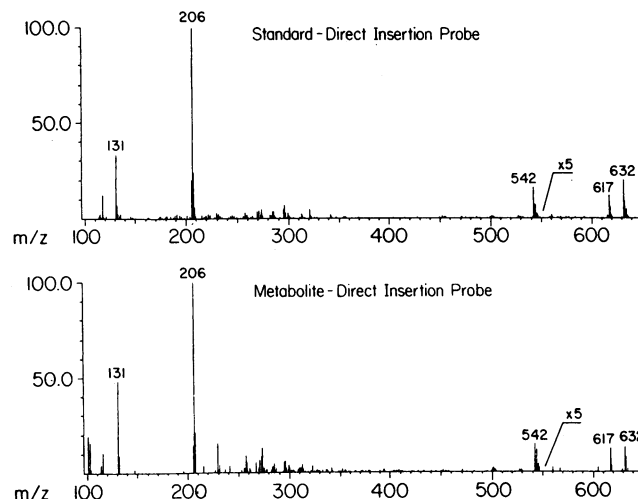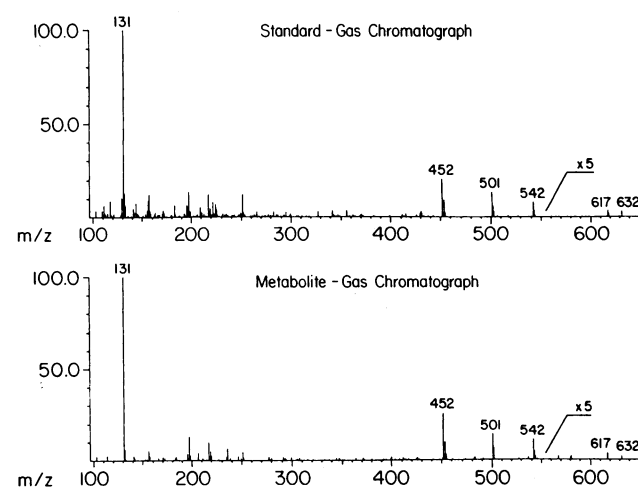‡ Significantly different from control at $P < 0.01$.

Figure 5. Electron impact (70 eV) mass spectra of the tris (trimethylsilyl) derivative (pyro form) of 1,25(OH)$_2$D$_3$ (top) and metabolite (bottom) after gas chromatographic separation (conditions given in Methods).

Discussion

The data presented here indicate that cord blood lymphocytes infected with HTLV-I, which behave in culture as HTLV-I–associated lymphoma cells, have the capacity to metabolize $25(OH)D_3$ to $1,25(OH)_2D_3$. It appears likely that hypercalcemia in HTLV-I–associated lymphoma may in part be caused by increased production of $1,25(OH)_2D$ by lymphoma cells.

Although the primary site of the 1-alpha-hydroxylase enzyme required for the conversion of $25(OH)D$ to $1,25(OH)_2D$ is the kidney, there is increasing evidence for extrarenal sites as well. 1-Alpha-hydroxylase activity has been reported in various tissues including the placenta (13, 14), chick chorion (15), rabbit and human bone cells (16, 17), melanoma cells (18), human pulmonary alveolar macrophages obtained from patients with sarcoidosis (19), and sarcoid granulomas (20). Sarcoidosis may be particularly relevant because there are many parallels between this condition and adult T cell lymphoma. Patients with sarcoidosis develop lymphadenopathy, and this disease tends to occur in clusters in the black population in the southeastern United States (21), as does adult T cell lymphoma associated with HTLV-I. In addition, patients with sarcoidosis may develop hypercalcemia with increased circulating concentrations of $1,25(OH)_2D$ (22). Sarcoidosis is characterized by abnormal T cell function and increased numbers of activated T lymphocytes of the mature helper-suppressor type in the lung (22). It is attractive to speculate that activated lymphocytes, in addition to pulmonary alveolar macrophages in patients with sarcoidosis, may contribute to the increased circulating concentrations of $1,25(OH)_2D$ seen in both of these diseases.

The clinical syndrome of T cell lymphoma characterized by hypercalcemia and the presence of antibodies to HTLV-I is of interest for several reasons. In contrast to nonvirus-associated lymphoma, these patients frequently have an aggressive, rapidly fatal course characterized by a paraneoplastic syndrome of increased bone turnover with hypercalcemia. One recent study reported that 5 of 11 patients with HTLV-I antibody–positive lymphoma presented with manifestations of hypercalcemia (1), and all 11 patients developed hypercalcemia at some time during the course of this disease. While some patients have had lytic bone lesions on radiographs, biopsy of these lesions has often failed to reveal tumor involvement and showed only increased osteoclast activity (2–5). There have been several well-documented cases in which no lytic lesions could be detected. Tumor extracts from one patient have been shown to cause bone resorption in mouse calvaria in vitro and to induce hypercalcemia in mice in vivo (23). These observations suggest that a humoral mechanism is involved in the pathogenesis of the hypercalcemia seen in HTLV-I–associated lymphoma. Hormones known to mediate bone resorption by increasing osteoclast activity have not been shown to be increased in this disease. Specifically, parathyroid hormone (PTH) levels are suppressed, and PTH has not been detected in tumor tissue obtained from these patients (23). Increased levels of prostaglandin E in blood or tumor tissue have not been found, and the hypercalcemia does not respond to the administration of indomethacin (23). Preliminary data from our laboratory suggest that osteoclast-activating factor (OAF), a polypeptide produced by lymphocytes that is capable of stimulating bone resorption in organ culture, may be produced by HTLV-I–transformed cells (24). Whether production of OAF is a consistent feature of HTLV-I–transformed lymphocytes requires further study.

$1,25(OH)_2D$ stimulates intestinal absorption of calcium in vivo and is also a potent bone-resorbing factor in vitro. Increased serum concentrations of this metabolite have been reported in seven patients with hypercalcemia and lymphoma. It is of particular interest that one of these patients was tested and found to be HTLV-I antibody–positive (3, 25, 26). It is not clear whether additional patients in these series may have had HTLV-I–associated lymphoma, since testing for the presence of antibodies to the HTLV-I virus was not reported. In addition, two patients with Hodgkin's disease and hypercalcemia have recently been described (26, 27). The finding of increased $1,25(OH)_2D$ levels in these patients in the presence of PTH suppression and significant renal impairment support the concept that lymphoma cells could produce $1,25(OH)_2D$ in sufficient amounts to stimulate increased intestinal absorption of calcium and increased bone turnover, thus resulting in hypercalcemia.

Our results clearly show that normal human lymphoid cells transformed by HTLV-I have the capacity to convert $25(OH)D_3$ into $1,25(OH)_2D_3$. While studies in normal rats (28) suggest that physiologically significant $1,25(OH)_2D$ production does not occur outside the kidney, it is possible that production of $1,25(OH)_2D$ in extrarenal sites occurs in pathological situations characterized by abnormal $1,25(OH)_2D$ levels. An interesting issue raised by these studies is whether or not these virus-transformed lymphocytes behave in a similar manner to normal peripheral blood lymphocytes that have been activated with an antigen or with phytohemagglutinin. Our experiments to date have shown no substantial production of a $1,25(OH)_2D_3$-like metabolite in normal peripheral blood leukocytes incubated with 1% phytohemagglutinin for 48 h. However, it remains possible that a small subset of normal T lymphocytes has the capacity to metabolize $25(OH)D_3$. Local production of even small amounts of $1,25(OH)_2D$ could have important effects on adjacent hematolymphopoietic or bone cells. Excessive production of $1,25(OH)_2D$ by lymphoma cells in vivo with subsequent osteoclast activation and increased intestinal calcium absorption should be considered as a potential pathogenetic factor in the hypercalcemia that occurs in patients with HTLV-I–associated T cell lymphoma.

Acknowledgments

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