Presence of osteoclast-like multinucleated giant cells in the bone and nonostotic lesions of Langerhans cell histiocytosis

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Langerhans cell histiocytosis (LCH) is a disease that can involve one or multiple organ systems characterized by an accumulation of CD1a$^+$ Langerhans-like cells as well as several other myeloid cell types. The precise origin and role of one of these populations, the multinucleated giant cell (MGC), in this disease remains unknown. This work shows that in three different lesional tissues, bone, skin, and lymph node, the MGCs expressed the characteristic osteoclast markers, tartrate-resistant acid phosphatase and vitronectin receptor, as well as the enzymes cathepsin K and matrix metalloproteinase-9. Although, in bone lesions, the osteoclast-like MGCs were only CD68$^+$, in the nonostotic sites, they coexpressed CD1a. The presence of osteoclast-like MGCs may be explained by the production of osteoclast-inducing cytokines such as receptor activator of nuclear factor κB ligand and macrophage colony-stimulating factor by both the CD1a$^+$ LCH cells and T cells in these lesions. As osteoclast-derived enzymes play a major role in tissue destruction, the osteoclast-like nature of MGCs in all LCH lesions makes them a potential target for the treatment of this disease.

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Langerhans cell (LC) histiocytosis (LCH) is a rare disease often present in childhood with a continuum of clinical entities ranging from a localized lytic lesion to a fatal disseminated myeloid-like leukemia and is associated with fibrosis and osteolysis, which leads to organ dysfunction (1). Although the pathophysiology is still obscure, at the cellular level, LCH is characterized by the clonal proliferation and retention of CD1a$^+$ dendritic LCs, commonly referred to as LCH cells. Together with LCH cells, other cell types have been shown to be present in LCH lesions, including lymphocytes, macrophages, eosinophils, and multinucleated giant cells (MGCs; reference 2).

MGCs are thought to originate from the fusion of monocyte–macrophage lineage cells (3). Morphologically, they can be classified into Langhans giant cells (normally found in infective granulomatous diseases; reference 4), foreign body giant cells (commonly found in foreign body granulomas; reference 5), or thirdly, osteoclasts, which are present in bone sites where they function in bone resorption (6). Although all these types of MGCs originate from a common precursor cell, they differ markedly in their association with disease states, location, and prevalence in various tissues or organs, stimuli that induce their formation, and subsequent function.

It is unclear how monocyte fusion is induced in vivo and whether different mechanisms are involved in different pathological states. However, a number of papers have reported on how the formation of MGCs can be induced in vitro. Evidence has accumulated to show that the in vitro generation of MGCs occurs as a result of cell fusion rather than cell division (7). In fact, the in vitro fusion of adherent macrophages from both humans and experimental animals is a normal event at a terminal stage of maturation (8). This phenomenon is enhanced, among other stimuli, by the addition of various cytokines. Indeed, the cytokines IL-4, IFN-γ, or IL-13 clearly play a prominent role in monocyte fusion and, subsequently, in the generation of MGCs (5, 9). Furthermore, an appropriate cytokine environment can regulate the commitment of a cell toward one or another cell lineage. For example,

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osteoclast differentiation from monocyte–macrophage precursor cells occurs in the presence of cytokines, such as TNF-α and IL-1α (10) or receptor activator of NF-κB ligand (RANKL), and growth factors, such as M-CSF (11). In contrast, although DCs originate from the same monocyte–macrophage precursor cells as osteoclasts, DCs are derived in vitro from circulating human monocytes after stimulation with GM-CSF, IFN-α, and IL-4 (12, 13), or from human $CD34^+$ myeloid progenitors in response to GM-CSF and TNF-α (14). These bone marrow progenitors were identified recently through their ability to differentiate into DCs or osteoclasts, depending on whether RANKL was present together with GM-CSF or M-CSF, respectively (15).

Thus, it is clear that the cellular environment plays a crucial role in cell differentiation. In this report, we demonstrate that the cytokine environment of LCH lesions may allow local formation rather than attraction of osteoclast-like MGCs. The local formation may explain the coexpression of CD1a observed on osteoclast-like MGCs in nonbone lesions as the normal osteoclast precursors are likely to be absent in these tissues. So, although the phenotype of the osteoclast-like MGCs was more normal in bone lesions, it seems likely that this population must contribute a large part of the chronic tissue destruction in all LCH lesions. Thus, the osteoclast-like nature of MGCs provides a rationale for the successful treatment of LCH patients with antiosteoclast therapy.

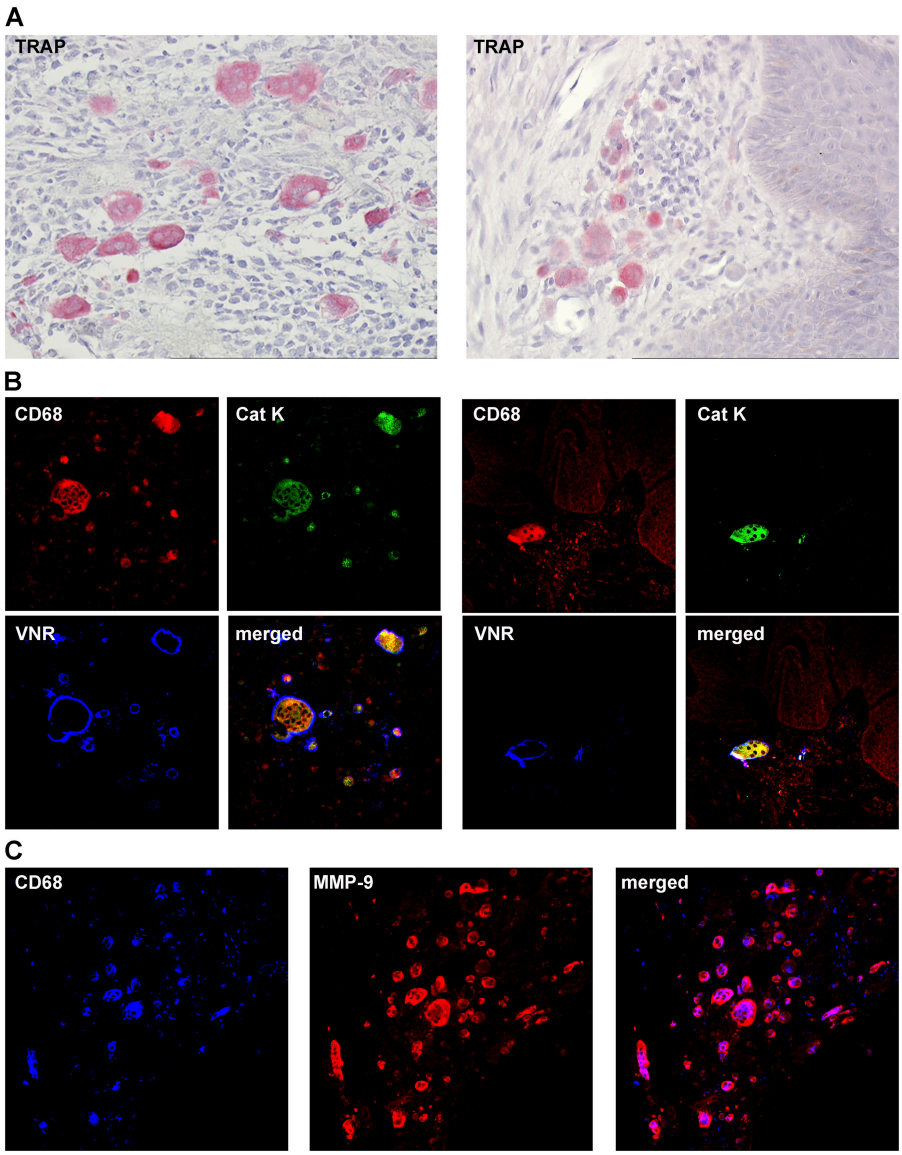

Figure 1. Phenotypic characterization of MGCs in LCH lesions for osteoclast markers. (A) MGCs in bone (left) and skin (right) LCH lesions were $TRAP^+$. (B) Triple-color immunofluorescent staining for CD68, CatK, and VNR in an LCH bone lesion (left) and a skin lesion (right). (C) Double immunofluorescent staining for CD68 and another osteoclast marker, MMP-9, in an LCH bone lesion. Original magnifications, (A) 220; (B) 290; and (C) 270.

RESULTS AND DISCUSSION

MGCs in LCH lesions phenotypically express osteoclast markers

Although the different types of MGCs all have a hematopoietic precursor, the osteoclast has very distinct functional and phenotypic characteristics (3). Thus, to clarify whether the MGCs observed in LCH lesions are indeed of an osteoclast-like phenotype, we performed multicolor immunohistochemical analysis for the typical osteoclast markers, CD68, tartrate-resistant acid phosphatase (TRAP), vitronectin receptor (VNR), and the enzymes cathepsin K (CatK) and matrix metalloproteinase–9 (MMP-9; Table I and Fig. 1). CD68, a marker of the monocyte–macrophage lineage cells, was used to detect MGCs in LCH lesions. $CD68^+$ MGCs were observed in 13 out of the 15 LCH bone biopsies analyzed. Importantly, MGCs were also found in nonostotic LCH lesional sites, namely the lymph node (4/4) and the skin (3/7). Five out of seven nonostotic lesions that contained MGCs stained positive for TRAP (Fig. 1 A, right), an enzyme present in osteoclastic vesicles that fuse with endocytic vesicles containing the bone matrix degradation products. This enzyme induces the release of reactive oxygen species that destroy the matrix components of the bone (16). 9 out of the 13 bone lesions also showed TRAP positivity on the $CD68^+$ MGCs (Fig. 1 A, left). Triple immunofluorescent staining for CD68, VNR, and CatK showed that all the bone (Fig. 1 B, left) and lymph node lesions (not depicted) with MGCs were VNR^+ and $CatK^+$. In contrast, one out of three skin lesions containing MGCs was positive for VNR and CatK (Fig. 1 B, right). A further enzyme characteristic of osteoclasts, MMP-9, was also present on the $CD68^+$ MGCs in all bone lesions (Fig. 1 C). Moreover, MMP-9 was also expressed in the MGCs of skin (1/3) and lymph node lesions (4/4). CatK and MMP-9 are proteases

Table I. Characterization of MGCs in LCH lesions for osteoclast markers, osteoclast-secreted enzymes, and osteoclast-inducing environment

	MGCs in lesion						Lesional environment		
		Phenotypic markers of osteoclasts			Osteoclast-secreting enzymes		Osteoclast-inducing cytokines and receptor		
Lesional site	CD1a	CD68	TRAP	VNR	CatK	MMP-9	M-CSF	RANKL	RANK
Bone									
b1	–	++	++	++	++	++	#	#	#
b2	–	++	–	++	++	++	–	#	#
b3	–	–	–	–	–	–	#	#	#
b4	–	+	–	+	+	+	#	#	#
b5	–	++	++	++	+	++	–	#	#
b6	–	+++	+++	+++	+	++	#	#	#
b7	–	++	++	++	++	++	#	–	#
b8	–	–	–	–	–	–	#	#	#
b9	–	++	–	+	+	++	#	–	#
b10	–	++	++	+	+	++	#	–	#
b11	–	+	+	+	+	+	#	#	#
b12	–	+++	+++	+++	+++	+++	#	–	#
b13	–	++	–	+	+	++	–	#	#
b14	–	+++	+++	++	++	++	#	#	#
b15	–	+	+	+	+	+++	–	#	#
Skin									
s1	++	++	++	++	++	+	#	#	#
s2	–	–	–	–	–	–	#	–	#
s3	–	–	–	–	–	–	ND	–	#
s4	–	–	–	–	–	–	ND	ND	–
s5	–	+	+	–	–	–	ND	ND	ND
s6	–	+	–	–	–	–	–	#	#
s7	–	–	–	–	–	–	–	–	–
Lymph node									
l1	++	++	++	++	++	++	#	#	ND
l2	ND	+	+	+	+	+	#	#	#
l3	–	+++	–	+++	+++	+++	#	#	#
l4	+	+	+	+	+	+	–	#	#

–, absence of expression; #, expression; +, 0–30% expression by the MGCs; ++, 30–70% expression by the MGCs; +++, 70–100% expression by the MGCs; ND, not determined due to lack of tissue.

involved in the degradation of organic components from the bone matrix, such as type I collagen and other matrix proteins (17, 18). VNR is a receptor for the integrin vitronectin commonly found in osteoclasts and likely to be involved in the interaction between the osteoclast and the bone matrix (19). Thus, the expression of typical osteoclast markers as well as characteristic osteoclast-secreted enzymes by the MGCs in LCH lesions confirms that these cells are indeed osteoclast-like MGCs.

Possible origin of MGCs in LCH lesions

The presence of these osteoclast-like giant cells in LCH bone lesions is perhaps not that unusual as this is the normal tissue site for osteoclasts, which, through their resorbing activity, help to maintain the normal homeostasis of the bone (6). However, even in the ostotic LCH lesions, these osteoclast-like cells were present in relatively higher numbers than in normal bone and appeared to be "floating" within the cellular infiltrate of the lesion, whereas normally close contact with bone would be expected. In contrast, the finding of osteoclast-like cells in nonostotic LCH sites raises the question of their origin. To investigate this, we performed triple immunofluorescent stainings for the LC marker, CD1a, the macrophage marker, CD68, and CatK to more clearly identify the MGCs. In all bone lesions, the $CatK^+$ osteoclast-like cells coexpressed the macrophage marker CD68. In none of the ostotic lesions did these osteoclast-like MGCs express CD1a (Table I and Fig. 2 A). This finding suggests that the MGCs in bone LCH display the features of a normal osteoclast. In contrast, in one out of three skin and two out of four lymph node lesions that contained osteoclast-like cells, the MGCs expressed both CD68 and CD1a (Table I and Fig. 2 B). Hence, although both the osteoclast-like giant cells in bone as well as in nonbone lesions expressed CD68, only the giant cells in skin and lymph node coexpressed CD1a. This unusual phenotype of these osteoclast-like giant cells in skin LCH has been reported before in a single case without any further characterization (20). The majority of nonostotic lesions studied were in fact from patients without additional bone lesions. This excludes the possibility that the MGCs were derived from bone lesions. Therefore, it is likely that the lesional environment induces the local formation of the osteoclast-like MGCs even in unusual sites, such as these nonostotic LCH sites. This, together with the fact that the normal precursors of osteoclasts are likely to be absent from these sites, may result in osteoclast-like MGCs derived from a different origin (e.g., $CD1a^+$ cells). Alternatively, the $CD1a^+$ expression by MGCs in these sites may be due to induced expression of CD1a at a later stage.

To better understand the likely mechanisms of MGC formation in LCH lesions, we looked at the expression of intercellular adhesion molecule-1 (ICAM-1), an adhesion molecule expressed by monocytes upon fusion to form MGCs (21) and Ki-67, a nuclear protein associated with somatic cell proliferation (22). We found that in all LCH lesions, the MGCs displayed strong membrane staining for ICAM-1. In contrast, the MGCs were consistently negative for the proliferative marker Ki67 (unpublished data). However, there was a high expression of Ki-67 in other cells in the lesions that we and others have shown previously to be largely due to the $CD1a^+$ LCH cells (23, 24). These findings suggest that the osteoclast-like MGCs present in LCH lesions may be formed by the fusion of resident monocytes–macrophages rather than by cell division. Cytokines such as IFN-γ, which has previously been shown to be expressed in LCH lesions (25), are well-known inducers of ICAM-1 expression and, thus, may initiate the fusion of monocytes and macrophages to form MGCs. Thus, MGCs seem to be intrinsic to LCH lesions and specific factors within the well-

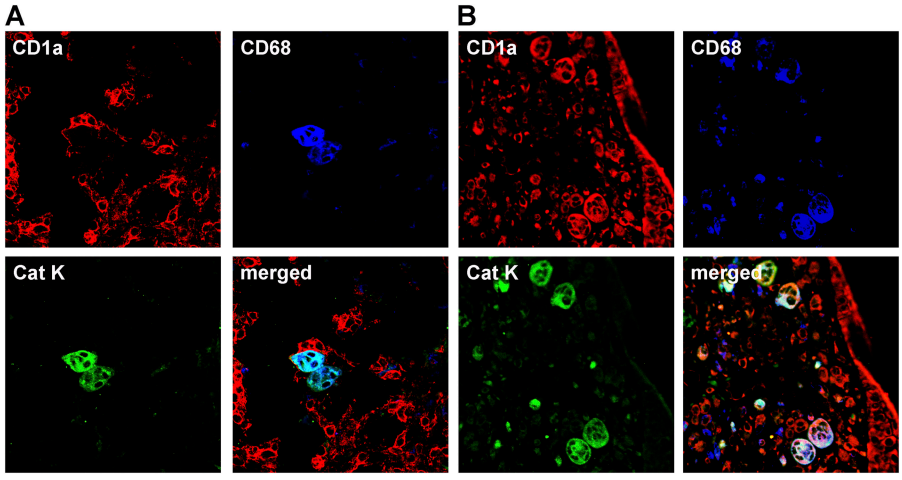

Figure 2. Phenotypic difference in osteoclast-like MGCs in bone versus nonbone lesions. Triple immunofluorescent staining for the monocyte lineage marker, CD68, DC marker, CD1a, and the enzyme CatK was performed in LCH bone (A) and skin (B). The osteoclasts in LCH skin lesions clearly expressed CD1a as well as CD68 and CatK (B). In contrast, LCH bone lesions never expressed CD1a (A). Original magnification, 260.

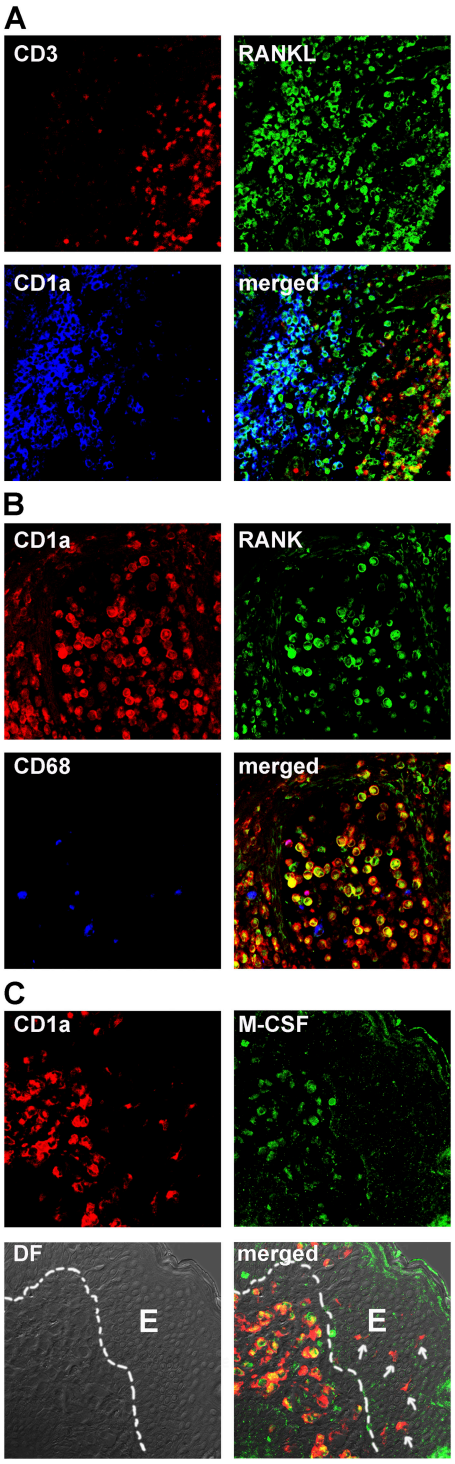

Figure 3. Expression of cytokines known to be involved in osteoclast differentiation in bone and skin LCH. Two- and three-color immunofluorescent stainings were performed for the cytokines RANKL and its receptor RANK and M-CSF. (A) Representative picture of an LCH bone lesion showing that the majority of the CD1a$^+$ LCH cells (blue) express RANKL (green). This colocalization results in a turquoise color. In addition, many of the neighboring T cells (red) also expressed RANKL (green). This colocalization resulted in a yellow color. (B) The CD1a$^+$ LCH cells (red) also expressed RANKR (green). This colocalization results in a yellow color in the merged image. (C) Representative picture of an LCH skin lesion showing that the osteoclast differentiation cytokine, M-CSF (green), was expressed by the CD1a$^+$ LCH cells (red). This colocalization resulted in a yellow color in the merged image. In contrast, normal LCs (indicated by arrows) in the epidermis (E) did not express any M-CSF. Original magnification, (A, B, and C) 270.

characterized "cytokine storm" in LCH lesions are responsible for their formation.

The osteoclast-inducing cytokines RANKL and M-CSF are highly expressed in LCH lesions

As shown by in vitro studies, the environment in which the mononuclear cells are present determines their differentiation into the various mononuclear phagocyte system-derived cells. Similarly, the tissue site and environment may have a large influence on the cellular composition of LCH lesions. Previous work by our group and others has clearly shown the presence of a cytokine storm in LCH lesions (25). For instance, factors involved in osteoclastogenesis such as IL-1, IL-6, and TNFα are highly expressed. In the present report, we have extended the analysis of cytokines to those specifically involved in the induction of osteoclast differentiation. One such cytokine involved in osteoclast induction is RANKL. In 24 LCH lesions studied for RANKL expression, 17 were found to be positive. We found that this cytokine was not expressed by the endothelial cells and macrophages, as assessed by triple staining combining RANKL with the CD31 and CD68 markers, respectively (unpublished data). Instead, triple staining for RANKL, CD1a to identify the LCH cells and the T cell marker CD3, revealed that the majority of CD1a$^+$ LCH cells and T cells in close proximity to the LCH cells expressed RANKL (Fig. 3 A). Thus, both the CD1a$^+$ LCH cells and T cells contribute to osteoclastogenesis through up-regulated RANKL and, thus, provide a mechanism for the potentiation of osteoclast formation and bone resorption in LCH lesions.

One key feature of osteoclast differentiation is the interaction between RANKL and its receptor, RANK, commonly expressed by the osteoclast precursor cells. We looked at the presence of RANK receptor on CD68$^+$ and CD1a$^+$ cells by triple immunofluorescent staining. All the lesions that showed expression of RANKL were also positive for RANK, which was expressed by a high proportion of CD1a$^+$ cells and to a lesser extent by CD68 cells as shown in Fig. 3 B. The expression of RANK by CD1a$^+$ cells as well as the presence of its ligand by activated T cells in LCH lesions is also important, as this interaction is known to induce a survival signal to DCs (26).

Furthermore, we looked at the expression of another cytokine known to be involved in osteoclast differentiation, M-CSF. M-CSF is normally produced by osteoblasts and/or stromal cells and is involved in the differentiation of osteoclasts from an early stage. We found it to be expressed by the MGCs and strikingly also by CD1a cells in 11 out of 15 LCH bone lesions. Interestingly, we found that this cytokine

was even expressed by the CD1a$^+$ cells in 1/3 skin (Fig. 3 C) and 3/4 lymph node lesions that contained MGCs. The expression of M-CSF by the lesional CD1a$^+$ cells seemed particularly relevant as there was clearly no expression of this cytokine by the normal LCs in both LCH skin lesions (Fig. 3 C) and normal skin (not depicted). The presence of cytokines involved in osteoclast differentiation in LCH lesions provides an explanation for the presence of osteoclast-like cells in ostotic as well as nonostotic sites in LCH. An attractive hypothesis would be that excessive amounts of osteoclast-inducing cytokines, such as RANKL and M-CSF, induce osteoclast-like differentiation of inappropriate precursors (e.g., CD1a$^+$ LCH cells). Strong support for this hypothesis was demonstrated in a very recent paper by Rivollier et al. (27). Using human monocyte-derived DCs generated in vitro, they showed that immature DCs can transdifferentiate into functional osteoclasts in the presence of M-CSF and RANKL. Furthermore, they showed that the proinflammatory cytokines, TNFα and IL-1α, both of which are highly expressed in LCH lesions (25), promote cell fusion during DC transdifferentiation and lead to larger MGCs than M-CSF plus RANKL alone. Thus, the in vitro differentiation plasticity seen in cells of the mononuclear phagocyte system also appears to occur in vivo in LCH lesions.

Rationale for the use of bisphosphonates in the treatment of LCH

Due to the lack of fresh biopsy material, it was not possible to perform functional studies such as the use of dentine discs to determine the resorbing capacity of the MGCs in LCH lesions. However, the finding that the MGCs in LCH lesions are expressing various matrix-degrading enzymes supports the hypothesis of a destructive role for these cells in LCH lesions. Such a role would also help to explain the predominant symptom of bone pain suffered by patients with LCH bone lesions. The present report has provided support for the hypothesis that the excessive bony destruction found in LCH is likely mediated by osteoclast-like giant cells. Therefore, these cells are a potential target in LCH lesions. To date, only a few case reports, including one we authored, have indicated the use of bisphosphonates as a successful treatment of bone LCH (28). However, all of these case reports lack the fundamental background for the rationale. Bisphosphonates appear to act when administered at therapeutic doses only in bone, which is probably due to their specific affinity to this tissue. This group of compounds is known to have an inhibitory effect on the number and activation of osteoclasts (29). Thus, this study has provided a rationale for the use of bisphosphonates in the treatment of LCH patients.

MATERIALS AND METHODS

Tissue samples. Representative specimens of formalin-fixed, paraffin-embedded tissue from 26 patients with a diagnosis of LCH were identified by immunohistochemistry using S-100 and CD1a as markers. All biopsies showed characteristic CD1a$^+$ LC cells, CD68$^+$ macrophages, CD3$^+$ T cells, and eosinophils. 15 specimens were obtained from bone in cases of ostotic LCH, 7 were obtained from skin biopsies in cases of isolated skin disease, and 4 were obtained from excisional lymph node biopsies from patients with solitary lymph node involvement. MGCs were seen in 20 out of the 26 cases. One tissue each of Paget's disease, dermatopathic lymphadenopathy, and normal skin were used as methodological controls to avoid false positive or negative stains due to technical flaws. Lesional tissue of Paget's disease of the bone, a disease characterized by the presence of activated osteoclasts, was used as positive control for the osteoclast-like MGCs of LCH lesions. Dermatopathic lymphadenopathy, a disease characterized by the accumulation of CD1a$^+$ DCs, but without MGCs and normal skin, was used as negative control. Experiments were approved by the ethical committee of Leiden University Medical Center.

Antibodies. All staining was done in PBS with 1% BSA. Mouse monoclonal antibodies to CD1a (1CA04), MMP-9 (4A3), and CD31 (JC/70A) were obtained from Neomarkers. Goat and rabbit polyclonal antibodies to CatK, M-CSF, and GM-CSF, respectively, were obtained from Santa Cruz Biotechnology, Inc. The mouse monoclonal to RANKL (70525) and the goat polyclonal to RANK were obtained from R&D Systems. The rabbit polyclonal to CD3 and the mouse monoclonal to Ki-67 (MIB-1) were obtained from DakoCytomation. The mouse monoclonals to VNR (CJ00) and ICAM-1 (23G12) were obtained from Novocastra. The mouse monoclonal to CD68 (514H12) was obtained from Serotec. Secondary antibodies for enzymatic staining were obtained from DakoCytomation, and substrate chemicals were obtained from Vector Laboratories. Secondary immunofluorescent reagents were goat anti–mouse, goat anti–rabbit, and donkey anti–goat isotype-specific Alexa Fluor antibodies (Molecular Probes).

Immunohistochemistry. The preparation of paraffin sections for staining was performed as described previously (30). Double and triple stainings with primary anticytokine, cytokine receptors, or osteoclast markers in combination with cell-specific markers were detected fluorescently using the relevant secondary goat anti–mouse, goat anti–rabbit, or donkey anti–goat isotype-specific Alexa Fluor 488, 647, or 546 secondary antibodies. Replacement of the primary antibodies by 1% PBS/BSA was used as a negative control. Results were analyzed by confocal microscopy using a confocal microscope in fluorescence and brightfield mode (LSM 510; Carl Zeiss MicroImaging, Inc.).

Single enzymatic staining for TRAP. TRAP staining was performed using a combination of solutions that include naphtol–AS BI phosphate, dimethylformamide, tartaric acid, acetate buffer, vermoal buffer, sodium nitrite, and pararoseaniline. Tissue sections were deparaffinized, rehydrated, and incubated with the reactive solution for 20 min. After washing with distilled water, the tissue sections were counterstained with Mayer's hematoxylin and mounted using Histomount media (National Diagnostics).

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