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The Role of Cytokines in the Pathogenesis of Langerhans Cell Histiocytosis

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Summary Langerhans cell histiocytosis (LCH) is characterised by an accumulation of cells ('LCH cells') with the same phenotypic features as normal Langerhans cells found in skin and other organs. The pathogenesis of LCH is unknown but there is increasing evidence to implicate the involvement of lymphokines and pro-inflammatory cytokines in the tissue damage seen in this disorder. Apart from histiocytes, the lesions contain giant cells, macrophages, neutrophils, eosinophils, lymphocytes, plasma cells and occasional mast cells that are the hallmark of an inflammatory process. The role of cytokines in the recruitment of haemopoietic cells within inflammatory lesions has only recently been recognised. In this article, we review the possible role of cytokines in the pathogenesis of LCH, and provide an overview of the methods currently used to detect and quantitate them. An appreciation of the type, distribution and amount of different cytokines released within lesions can provide clues to the possible aetiology of LCH. Using immunoassays, *in situ* hybridisation and RT-PCR, increased amounts of IL-1, IL-3, IL-4, IL-8, GM-CSF, TNFα, TGFβ and LIF have been demonstrated in LCH lesions. Lymphocytes constitutively produce GM-CSF and IL-3 and, to a lesser degree, IL-1, IL-4 and LIF whilst histiocytes produce TNFα, IL-1β and GM-CSF.

Introduction

Langerhans cell histiocytosis (LCH) is a disorder of unknown aetiology, characterised by an accumulation, in a variety of organs, of cells with the same phenotype as normal epidermal Langerhans cells ('LCH cells') together with inflammatory cells -eosinophils, lymphocytes, neutrophils, macrophages, plasma cells, B cells, mast cells and giant cells. In decreasing order of frequency, LCH can involve bone, skin, gastrointestinal tract including mouth, liver, spleen, lung, lymph nodes, bone marrow and central nervous system (CNS), particularly the hypothalamus/pituitary and cerebellum (see Broadbent *et al.*, this volume). Clinical observations, especially the high rate of spontaneous remission and the histopathological appearances, which do not have the classical hallmarks of malignancy, have led many observers to regard LCH as a 'reactive' disease, often running a chronic relapsing course. Such reactions are often secondary to alterations in cytokine production. The histological features of LCH also suggest that cytokines may be involved in the pathogenesis of LCH. The aims of this chapter will be (a) to provide an introduction to the properties of cytokines with an emphasis on the role of cytokines in inflammation and in LCH and (b) to suggest how this knowledge may be used to formulate better treatment strategies aimed at reducing tissue damage in patients with LCH.

General properties of cytokines

Cytokines are protein hormones produced by the cells of the immune and inflammatory systems. Their principal functions are to mediate and regulate immune and inflammatory reactions, and to provide communication between leukocytes and between leukocytes and other cell populations (Paul & Seder, 1994). The production of cytokines is usually brief, is initiated by new gene transcription, and is triggered by specific stimuli, such as endotoxin for macrophages and protein antigens for T lymphocytes. Cytokines act on cells that express specific, high-affinity receptors and initiate a wide variety of biological responses. Most cytokine actions are autocrine or paracrine; however, since macrophages can produce large amounts of cytokines in severe infections, macrophage-derived cytokines can also have endocrine actions, distant from the sites of production. Cytokines are pleiotropic, ie. any one cytokine has numerous effects, and redundant, ie. multiple cytokines may initiate the same biologic response. One cytokine can stimulate production of another. Such 'cascades' provide a mechanism for amplifying biological responses in physiological conditions, and often contribute to the development of clinico-pathological abnormalities. Different cytokines may also be synergistic or antagonistic in their actions.

Cytokines may be classified into several categories based on their principal functions. This provides a useful framework for analysis, but it is clear that such classifications are imperfect, because there may be substantial overlaps between the groups. The **mediators of inflammation** (proinflammatory cytokines) are produced mainly by macrophages; endothelial cells, T lymphocytes, and Langerhans cells are other sources of these cytokines, which include TNFα, IL-1, IL-6 and the family of chemokines. These cytokines are responsible for the recruitment and activation of leukocytes, giving rise to acute inflammation at sites of infection, and for the systemic manifestations of inflammation. The **regulators of lymphocyte growth and differentiation** are produced mainly by CD4+ helper T lymphocytes. This group includes IL-2, IL-4 and IL-10. These cytokines stimulate and regulate lymphocytes, and are thus involved in the development, or 'afferent', phase of specific immune responses. Recently discovered members of this group include IL-13 (which functionally resembles IL-4), IL-14 (a B cell growth factor), and IL-15 (whose actions are similar to those of IL-2). **Activators of specialised effector cells** are produced mainly by T lymphocytes; some are also secreted by macrophages. They include IFNγ (the principle macrophage-activating cytokine), IL-5 (the eosinophil-activating cytokine), and IL-12 (which stimulates natural killer cells and cytolytic T lymphocytes). These cytokines enable antigen- stimulated lymphocytes to trigger the effector systems that serve to eliminate the antigen, and are thus the mediators of the 'efferent' phase of immune responses. Finally, a variety of **haemopoietic cytokines**, including colony-stimulating factors, IL-7 and IL-11, are produced by T lymphocytes, macrophages, and stromal cells in the bone marrow. These cytokines function to replenish the leukocytes that are consumed during immune and inflammatory reactions.

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Methods for detecting cytokines

Biological assays

The detection of various cytokines produced by cells in conditioned media or released into tissue fluids can be implied by observing their effect on a biological system. These assays can be based on a number of biological 'endpoints' such as cell proliferation, cytotoxicity, colony formation, or induction of other cytokines or proteins. The targets for bioassays are usually primary cultures of cells such as haemopoietic progenitor cells or cytokine-dependent cell-lines. However, these assays are rarely specific and it is essential to use monospecific neutralising antibodies to confirm the identity of particular cytokines. Although bioassays identify biologically active molecules, and are probably the most sensitive assays for cytokines available to date, the difficulty of performing and interpreting these assays has led to attempts to develop alternative methods. An overview of quantitative biological assays for individual cytokines can be found in Wadhwa *et al.*, (1991).

Immunoassays

Immunoassays are based on the use of antibodies specific for individual cytokines. These assays are generally sensitive, reliable, and quick. There are three main types of immunoassays; radioimmunoassay (RIA), immunoradiometric assay (IRMA) and enzyme-linked immunoabsorbent assay (ELISA). The sensitivity of these assays is currently in the order of 1–10 pg/ml and they are widely used by many investigators to detect various cytokines secreted into serum, body fluids and supernatants of cultured cells. A failure to detect cytokines by bioassays or immunoassays in serum, body fluids, or conditioned media has to be interpreted with some caution as several studies have shown that polarisation of receptors occurs in response to cytokine release. This may lead to preferential binding of cytokines to activated cells in the surrounding microenvironment, with little or no release into the circulation (Poo *et al.*, 1988; Kupfer *et al.*, 1991).

Cytokine production by individual cells

The production and accumulation of cytokines by cells within lesions has been difficult to detect and quantitate as many of these molecules bind to high-affinity cellular receptors, are localised to the external surfaces of the cell(s) or are rapidly destroyed. Immunohistochemical techniques have been used to identify a number of cytokines in well preserved tissues, using the standard avidin-biotin or APAAP techniques (see Lewis, 1991). This technique appears to detect predominantly intracellular cytokines and not membrane-associated cytokines (Bogen *et al.*, 1993). Recently, immunofluoresence methods have also been used to identify and quantify cytokine-producing cells in tissue sections and cell suspensions (Sander *et al.*, 1991; Nagy *et al.*, 1993). A modification of the ELISA method, termed the ELISPOT technique, has also been used to identify and quantify cytokine-producing cells in cell suspensions.

Qualitative and quantitative cytokine mRNA analysis

Expression of cytokine mRNAs has traditionally been performed using the northern blot technique, by which the size of the mRNA transcript can be determined. However, this technique is insensitive and requires significant starting amounts of non-degraded RNA (5–10 μg polyA+ mRNA). The problem is further compounded by the fact that in many inflammatory tissues RNA is often rapidly degraded. The sensitivity of mRNA detection can be increased at least 10-fold by using the RNase protection assay, but still requires the transfer and hybridisation of each mRNA with specific radioactive probes. Two major problems in the study of cytokine mRNAs in LCH and related disorders are, first, the difficulty in obtaining enough material from which to extract RNA and, second – as with many other inflammatory conditions – RNA degradation, which makes standard northern analysis difficult to interpret. The advent of polymerase chain reaction (PCR) technology means that most of these problems can be circumvented. The refinement of reverse-transcription PCR (RT-PCR) facilitates rapid detection of multiple cytokine mRNAs from small numbers of cells (less than 1 ng of total RNA) (Brenner *et al.*, 1989). RT-PCR involves isolation of RNA from between $1-10^6$ cells, followed by reverse transcription of total RNA to cDNA, then amplification of specific DNA fragments using PCR. However, the standard RT-PCR approach only yields qualitative results. Recently a number of investigators (Wang *et al.*, 1989; Gilliland *et al.*, 1990) have reported modifications of this technique for quantitation of cytokine mRNAs. In general, these approaches either involve the use of synthesised internal standards so that reverse transcription and coamplification of the RT products occur simultaneously (Wang *et al.*, 1989), or the use of a competitive reaction between an added competitor DNA fragment which has been altered by the addition or deletion of nucleotide sequences (Gilliland *et al.*, 1991).

In situ *hybridisation*

In situ hybridisation has become a powerful and versatile tool for the localisation and detection of DNA and RNA sequences within cell or tissue preparations. Radiolabelled probes have mostly been used for mRNA detection, either as single-stranded RNA probes (Cox *et al.*, 1984), or as synthetic oligonucleotide probes (Coghlan, 1985). The realisation that oligonucleotide probes could be used to detect mRNA species in routine formalin-fixed paraffin sections has enabled this technique to be applied to the study of cytokine gene expression in lesions from patients with LCH (Kannourakis G, Nouri S, Iannello P, Wood J, and Tiong, T, unpublished observations).

The available techniques for detecting cytokines in clinical samples, such as body fluids or cells, are now relatively sensitive and reliable but each technique also has significant limitations. A combination of a morphological method, such as *in situ* hybridisation or immunohistochemistry, with a quantitative RT-PCR assay appears to be the best approach for detecting cytokines in tissues and cell suspensions. Immunoassays are the most reliable for quantitating cytokines in body fluids and culture supernatants.

Cytokine studies in LCH

1. The principal questions

The goal of studying the production of cytokines in LCH is to improve our understanding of the pathogenesis of the disease and, ultimately, to explore the therapeutic potential of recombinant cytokines and specific antagonists in this disorder. To date, most studies have focused on descriptive analyses of cytokine production in LCH lesions, delineation of which cells in the lesions produce which cytokines, and measurement of cytokine levels in patients sera. Such studies may lead to answers to 3 main questions:

i) Is the proliferation of cells in LCH lesions due to the local secretion of cytokines that act as autocrine and/or paracrine growth factors? Whether LCH is a clonal disorder or an exaggerated polyclonal cellular response, uncontrolled cellular proliferation may be stimulated by locally produced cytokines. The realisation that GM-CSF and TNFα stimulate the growth of normal Langerhans cells *in vitro* has led to particular interest in these cytokines in LCH lesions.

ii) Is the cellular infiltrate of LCH due to local cytokine production? Cytokines, such as TNF and IL-1, enhance the expression of adhesion molecules on vascular endothelium and promote the local accumulation of leukocytes. IL-4 is particularly potent at inducing eosinophil accumulation. Locally produced cytokines may also stim-

ulate the promotion and activation of cells that infiltrate the lesions. For instance, IL-5 is a potent eosinophil-activating cytokine, and may contribute to the eosinophil-rich infiltrate of eosinophilic granulomas.

iii) Are any clinical manifestations of LCH due to cytokines? This possibility is raised because of the known systemic effects of macrophage- derived cytokines, such as TNF, IL-1 and IL-6, in acute and chronic inflammation. In addition, cytokines such as TNF are capable of injuring a variety of tissues, including epithelial cells and cells of the central nervous system. Others, such as IL-4 and IL-10, suppress cell-mediated immunity, and could contribute to increased susceptibility of patients to infection.

2. Detection of cytokines in serum of patients with LCH

There is only one published study of cytokine activity in the sera of patients with active LCH. Komp and her colleagues showed that serum levels of TNFα, IL-2, and soluble IL-2 receptors were not elevated in LCH patients with active disease but were raised in the sera of patients with Hemophagocytic Histiocytosis syndromes ('HLH') compared to age-matched controls (Komp *et al.*, 1989). We have now used both immunoassays and bioassays to analyse sera from patients with active LCH, and have not detected high levels of IL-1, IL-3, IL-4, GM-CSF, TNFα, IL-8 or LIF. Of 14 samples examined 2 had significantly elevated IL-1 levels (>40 pg/ml), 3 had detectable IL-3 (13–95 pg/ml), none had elevated IL-4 levels, 2 had significantly detectable GM-CSF (8-22 pg/ml), 2 had slightly elevated TNFα (55–80 pg/ml), 6 had detectable IL-8 levels (45–2100 pg/ml) and none had detectable LIF levels (Kannourakis G, *et al.*, unpublished observations). A surprising finding was that a few patients with severe multisystem disease had detectable GM-CSF and IL-3 levels, implying that some of these cytokines may be released into the circulation. Other studies have confirmed high levels of circulating IL-6, IFNγ, and soluble IL-2 receptor in patients with hemophagocytic lymphohistiocytosis (HLH) (Fujiwara *et al.*, 1993).

3. Constitutive production of cytokines from cultured LCH mononuclear cells

A number of studies have revealed the constitutive production of IL-1 by normal and activated Langerhans cells from skin (Sauder *et al.*, 1984; Enk & Katz, 1992). It is also known that, after PMA and LPS stimulation *in vitro*, Langerhans cells produce TNFα and IL-1 (Larrick *et al.*, 1989). By contrast very few 'cytokine studies' have been performed on samples from LCH lesions. In one such study, FACS-sorted CD1a+ cells from eosinophilic granulomas were shown to produce both IL-1 and PGE2 in culture (Arenzana-Seisdedos *et al.*, 1986). More recent studies have demonstrated that in media conditioned by mononuclear cells obtained from eosinophilic granulomas, IL-1, TNFα, GM-CSF, IL-8, and LIF were detectable in significant quantities by immunoassay and biological assays. IL-1 was detected in 14/15 conditioned media (range 0->1,000 pg/ml), IL-8 in 15/15 (1,300– 4,800 pg/ml), GM-CSF in 15/15 (160–2,500 pg/ml), TNFα in 9/15 (0–4,800 pg/ml), IL-3 in 4/15 (0–15 pg/ml), and LIF in 6/6 (3.5–90 ng/ml), whereas IL-4 was only barely detectable in 2/15 samples (Kannourakis *et al.*, manuscript in preparation). This pattern of constitutive cytokine production by mononuclear cells from LCH lesions implicates the proinflammatory cytokines IL-1, IL-8, TNFα, GM-CSF and LIF in the pathogenesis of LCH. It is interesting that no IL-2, IFN, IL-5, or IL-6 were detected by immunoassay in LCH conditioned media. IL3 and IL4 were barely detected but may be present in biologically potent quantities because as little as a few pg/ml of these factors are capable of maximally stimulating target cells. Indeed, biologically active IL-3 was detected in media conditioned by LCH cells, and this activity was abrogated by neutralising antibodies to IL-3 (Kannourakis *et al.*, manuscript in preparation).

4. Detection of cytokine mRNA in LCH lesions

IL-1, IL-3, IL-4, IL-8, GM-CSF, TNFα, TGFβ, and LIF mRNA transcripts have been detected, by RT-PCR, from RNA obtained from LCH lesions (Kannourakis *et al.*, manuscript in preparation). No transcripts for IL-2, IL-5, IFNγ, or IL-6 were detected by RT-PCR. The lesions studied included eosinophilic granuloma, and skin lesions. The specificity of each amplified band was determined by probing with specific internal radioactive oligonucleotides. The detection of 2 prominent transcripts for IL-4 in LCH lesions is of interest. Recently, Sorg *et al.*, (1993), have identified 2 IL-4 mRNA transcripts from PHA-stimulated normal lymphocytes, and have shown that the smaller, less abundant species is an alternatively spliced transcript lacking the sequence encoded by exon 2. The significance of the prominent alternatively-spliced transcript is uncertain at present but it could conceivably encode for an IL-4 'antagonist' protein *in vivo*. Similarly, GM-CSF was also detected as 2 transcripts within LCH lesions, but only one transcript was detected by RT-PCR of PMA/ionomycin-stimulated normal lymphocytes (Kannourakis G, *et al.*, unpublished observations). Alternatively spliced transcripts for IL-2, IL-5 and G-CSF (Sorg *et al.*, 1991) and for M-CSF (Cosman *et al.*, 1988) have been identified but the significance of the second GM-CSF transcript in LCH is unclear.

These findings have been confirmed by *in situ* hybridisation using radiolabelled anti-sense oligonucleotide probes. Lymphocyte-like cells were positive for GM-CSF, IL-3, LIF, IL-4 and, to a lesser degree, TNFα and IL-1. Similarly, histiocytes were positive for IL-1, TNFα, IL-8, TGFβ and LIF. No hybridisation was detected with IL-2, IL-5, or IFNγ anti-sense probes (Kannourakis G, *et al.*, manuscript in preparation). The presence of abundant IL-4 mRNA, detected by RT-PCR, and *in situ* hybridisation in LCH lesions coupled with the finding of very low or no protein by immunoassays suggests that this species is actively synthesised in LCH lesions but may be membrane-bound rather than secreted. Eosinophils did not appear to be producing detectable mRNA for any of these cytokines. Giant cells were positive for IL-1, TNFα, LIF, GM-CSF and, to a lesser extent, IL-4 mRNA.

The pathogenesis of LCH: hypothesis and future directions

The patterns of cytokines detected in LCH lesions are consistent with local activation of T lymphocytes and other, inflammatory leukocytes. The T lymphocytes present within LCH lesions appear to be polyclonal (Kannourakis G, unpublished observations: Willman C, personal communication) and the profile of constitutively produced cytokines suggests that these cells are 'activated'. Constitutive production of both IL-3 and GM-CSF, in an experimental setting, is very unusual. One such setting, however, is the stimulation of polyclonal Vβ-restricted T lymphocytes by superantigens, such as Staphylococcal enterotoxins, leading to the release of IL-3, GM-CSF, IL-8, LIF and TNFα (Andersson *et al.*, 1992). This, in turn, could lead to the attraction of neutrophils, eosinophils, macrophages and plasma cells and circulating CD34-positive cells capable of proliferating and differentiating to CD1a-positive Langerhans cells with the same phenotypic and ultrastructural features as 'LCH cells' (Schmitt D, personal communication). Furthermore, the presence of both IL-4 and LIF within these lesions may also account for the presence of multinucleated giant cells within LCH lesions. Local production of IL-1 and LIF would be a potent stimulus to bone resorption, producing the characteristic radiological bony changes seen in LCH.

This hypothesis may help to explain the pathogenesis at one particular point in time but it does not explain how the disease waxes and wanes with time, nor does it take into account the chronicity and the eventual 'burning out' of the disease in many patients. Parallels could be drawn with autoimmune disease processes and the challenge is to devise testable models that take these observations into account.

Animal models may be especially useful for testing cytokines and specific antagonists, but to date such models have not been developed. Precise identification of the nature and cellular sources of cytokines, changes with disease remission and exacerbation, and effects of therapy should be important objectives of continuing studies. Such analyses may lead to strategies for combining 'biological response modifiers' with other treatment modalities in LCH. For instance, it may be possible to use drugs such as cyclosporin (Mahmoud *et al.*, 1991), which blocks the transcription of numerous cytokine genes, intravenous γ-globulin, whose mode of action is unknown but may be via inhibition of pro-inflammatory cytokines (Newberger *et al.*, 1986), or specific cytokine antagonists to control tissue destruction and inflammation in LCH.

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