Interleukin-1 Alpha- and Beta-, Interleukin-6 and Tumour Necrosis Factor-alpha-like Immunoreactivities in Chronic Granulomatous Skin Conditions

AHMED ABDELAZIZ AHMED¹, KLAS NORDLING¹, MARIANNE SCHULTZBERG² and STURE LIDÉN¹

¹Department of Dermatology, Karolinska Hospital, Stockholm and ²Department of Clinical Neuroscience and Family Medicine, Geriatric Medicine, Karolinska Institute, Novum, KFC, Huddinge, Sweden

Paraformaldehyde-fixed tissue of chronic granulomatous skin conditions, such as cutaneous leishmaniasis, granuloma annulare, leprosy and hidradenitis, was investigated for the presence of interleukin-1 alpha-, interleukin-1 beta-, interleukin-6 and tumour necrosis factor-alpha-like immunoreactivities among the cellular infiltrates. There was a weak to strong cytoplasmic labelling of plasma cells for interleukin-6 and tumour necrosis factor-alpha at the periphery of the granulomatous mass and around the skin appendages. The interleukin-6-like immunoreactivity seemed to be correlated with the coarseness of the chromatin material of the cells, being more intense with coarse chromatin. The cytoplasmic labelling for interleukin-1 alpha and interleukin-1 beta in the plasma cells was less intense. Epitheloid, Langhans's giant cells and small round cells exhibited a weak to moderate cytoplasmic labelling for interleukin-1 alpha and interleukin-1 beta, whereas the staining intensity for interleukin-6 and tumour necrosis factor-alpha was weak to strong. In addition, there was staining of the stroma in the centre of granuloma with antiserum against interleukin-1 alpha, interleukin-1 beta, interleukin-6 and tumour necrosis factor-alpha. This area contained few cells, suggesting that the granuloma was in a resolution process.

A contribution of interleukin-6 and tumour necrosis factor-alpha to the granulomatous reaction, at least during the maintenance period, is suggested by the occurrence of these cytokines in the skin conditions studied. The findings are also consistent with a suggested role of B cells in the late stages of the granulomatous reaction. In addition, they are in line with the reported declining role of interleukin-1 in the maintenance of granuloma.

Key words: cutaneous leishmaniasis; hidradenitis; granuloma annulare; leprosy; plasma cells; immunohistochemistry.

(Accepted May 4, 1994.)


A.A. Ahmed, Department of Dermatology, Karolinska Hospital, Box 120, S-171 76 Stockholm, Sweden.

The formation of a distinctive tissue mass known as a granuloma is a central event of a wide range of diseases affecting animals and man. Many of these diseases have cutaneous manifestations (1). The chronic granulomatous inflammatory diseases result in tissue destruction and end stage fibrosis (2). Numerous agents such as bacteria, fungi, helminthes and viruses, some metals, e.g. beryllium, and complex dyes, cause granulomatous inflammation. In addition, there are a number of granulomatous diseases of unknown aetiology (3).

Besides the broad classification of granulomatous inflammations into non-immunological or foreign body and immunological types, each with its characteristic histological criteria (1, 3), there is a further classification into two main families according to the presence or absence of B cells within the granuloma (4).

Granulomatous inflammation is characterized by a heterogeneous cellular infiltrate during the lesional development, among which the individual cells are important constituents. These cells are mainly T lymphocytes, plasma cells, macrophages/monocytes, epitheloid and Langhans giant cells (1–3). Recent studies have suggested a role for B cells in the formation and maintenance of the granuloma (5, 6). In addition, B cells were observed around the epitheloid cell aggregates in the late reaction of the granuloma (7).

It is of importance to elucidate the different roles of various cytokines or protein signals that are generated by different types of infiltrating cells. Some cytokines, like interleukin (IL)-1, IL-2, interferons and migration inhibitory factors, have been found in granuloma (8). A recent report stated that IL-2 and IL-4, as part of a cytokine cascade, were involved in granuloma formation in murine schistosomiasis, while interferon-gamma antagonized their production and the granulomatous response (9).

The skin does not only constitute a simple mechanical barrier, but upon injury it may be activated to release cytokines from keratinocytes, actively participating in repair mechanisms and elimination of injurious agents (10). These include IL-1, IL-6 and TNF-alpha, which are typical examples of multifunctional cytokines (11), produced by leucocytes in normal tissue and in a variety of inflammatory conditions (12,13). These cytokines have also been demonstrated in other cell types, e.g. fibroblasts, endothelial cells (14), adrenal chromaffin cells (16), bone marrow stromal cells and brain astrocytes (12), and there is a wide range of interactions between these cytokines as well as with other cytokines (15). A variety of biological actions on immune and non-immune cells have been reported (12).

The cellular distribution of IL-1 alpha, IL-1 beta, IL-6 and TNF-alpha in different chronic granulomatous skin conditions was investigated by immunohistochemistry in an attempt to understand their possible role in this reaction.

MATERIAL AND METHODS

Skin biopsy specimens

Biopsy specimens were taken under local anaesthesia from lesional skin of untreated patients with cutaneous leishmaniasis (n = 10), granuloma annulare (n = 10), leprosy (n = 6) and hidradenitis (n = 10). The duration of the clinical history was at least 6 months. The specimens were fixed in 4% buffered paraformaldehyde for 12–24 h, embedded in paraffin wax and stored until use.
Fig. 1. Immunoperoxidase micrographs of paraffin-embedded sections of human cutaneous leishmaniasis (a-g) and hidradenitis (h and i) after incubation with antibodies to IL-1 alpha (a and b), IL-1 beta (c), IL-6 (e and g) and TNF-alpha (d, f, h and i). Weak staining for IL-1 alpha (a) and IL-1 beta (c) can be seen in plasma cells (arrows) and epithelial cells (small arrow-heads) in cutaneous leishmaniasis, and weak IL-1 beta immunoreactivity is also seen in a Langhans giant cell (big arrow-head in c). (b) shows the lack of cellular staining after incubation with the IL-1 antiseraum preabsorbed with rmIL-1 alpha. Intense cytoplasmic labelling for TNF-alpha and IL-6 is observed in Langhans giant cells (big arrow-heads) and epithelial cells (small arrow-heads) in d and g, and plasma cells have strong cytoplasmic staining for IL-6 and TNF-alpha (arrows) in e and h (cutaneous leishmaniasis and hidradenitis). Note staining for TNF-alpha in the plasma cells (arrow) in f (cutaneous leishmaniasis), which seems to be membrane-bound. Stars in g and i indicate stromal staining for IL-6 and TNF-alpha, respectively (cutaneous leishmaniasis and hidradenitis). Magnifications are 400 × (a–d, f, g, i) and 1000 × (e and h).

Acta Derm Venereol (Stockh) 74
Table I. Cytokine staining intensity of cells in chronic granulomatous skin conditions

<table>
<thead>
<tr>
<th>Cell type</th>
<th>IL-1 α</th>
<th>IL-1 β</th>
<th>IL-6</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma cells</td>
<td>+ to ++</td>
<td>+ to ++</td>
<td>+ to ++</td>
<td>+ to ++</td>
</tr>
<tr>
<td>Epitheloid cells</td>
<td>+ to ++</td>
<td>+ to ++</td>
<td>+ to ++</td>
<td>+ to ++</td>
</tr>
<tr>
<td>Langhans giant cells</td>
<td>+ to ++</td>
<td>+ to ++</td>
<td>+ to ++</td>
<td>+ to ++</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>-</td>
<td>+ to ++</td>
<td>+ to ++</td>
<td>+ to ++</td>
</tr>
<tr>
<td>Small round cells</td>
<td>+ to ++</td>
<td>+ to ++</td>
<td>+ to ++</td>
<td>+ to ++</td>
</tr>
</tbody>
</table>

Reagents

Polyclonal antisera to IL-1 alpha, IL-1 beta and TNF-alpha were raised in rabbits and appropriately diluted in a solution of 0.25% bovine serum albumin (BSA) containing 0.01% NaN₃, in phosphate-buffered saline (PBS), pH 7.4. These antisera were kindly provided by Dr. Stefan Syversen (The Swedish Institute for Infectious Disease Control, Stockholm). The antisera were then applied at dilutions of 1:400 (IL-1 alpha), 1:400 and 1:800 (IL-1 beta, 1:200 and 1:400 (TNF-alpha), respectively (for details see references 16, 17). In addition, a polyclonal rabbit TNF-alpha antisera (Genzyme, Boston, MA, USA) was used at a dilution of 1:200, and a monoclonal mouse IL-6 antiserum directed against denatured IL-6 (Cappel, Reece, Belgium) was used at 1:50. Peroxidase-conjugated rabbit anti-mouse IgG and swine anti-rabbit IgG (Dako, Copenhagen, Denmark) were used at a dilution of 1:15. The substrate 3-amino-9-ethylcarbazole was purchased from Sigma (St. Louis, MO, USA).

Recombinant murine (rm) IL-1 alpha (115-270), R&D 4466 (a generous gift from Dr. P. L. Lommedico, Hoffmann-La Roche, Nutley, NJ, USA), with a specific activity of 1.3 x 10⁵ U/mg, was used at a final concentration of 6.4 µg/ml. Recombinant human (rh) IL-1 beta was a kind gift from Dr. Jesper Brustad (Department of Neurochemistry and Neurotoxicology, Stockholm University), and used at 100 µg/ml, rIL-6 (Boehringer Mannheim, Germany), with a specific activity of 2.5 x 10⁵ U/mg, was applied at a final concentration of 0.02 µg/ml, after being boiled for 40 min at 100°C. rTNF-alpha (BioSource International, Camarillo, CA, USA), with >95% purity, as detected by SDS-PAGE and committed N-terminal sequencing analysis, or rTNF-alpha (MI 148) (a kind gift from Reanal, Budapest, Hungary) was used at a final concentration of 5 µg/ml.

Immunohistochemistry

Sections (4.5 µm thickness) of the skin biopsies were mounted on gelatin-coated slides and incubated for 1 h at 60°C in order to melt the paraffin. Deparaffinization was performed in Bio-Clear (Bio-Optica, Milano, Italy) for 10–15 min and the sections were rehydrated in ethanol (100, 95 and 70%). Endogenous peroxidase activity was blocked subsequently by immersing the sections in a freshly prepared mixture of concentrated methanol and hydrogen peroxide (0.5%) for 10–20 min. The sections were then incubated with primary antibodies overnight at 4°C, soaked in PBS and incubated with peroxidase-conjugated secondary antibodies, anti-mouse IgG or anti-rabbit IgG, for 30 min. Three-amino-9-ethylcarbazole was used as chromogen. Finally, the sections were counterstained with haematoxylin for 5 min.

Specificity control

To confirm the specificity of the monoclonal and polyclonal antibodies for their respective antigen, the antibodies were preincubated over night at 4°C either alone or in the presence of the relevant antigen, i.e. monoclonal anti-IL-6 with rIL-6, and polyclonal anti-IL-1 alpha with rIL-1 alpha, etc. The preabsorbed solutions were centrifuged at 10,000 g for 5 min before use. Additional control sections were incubated with PBS instead of primary antibodies.

Analysis of staining

The assessment of the immunolabelling for IL-1 alpha, IL-1 beta, IL-6 and TNF-alpha was performed in a light microscope (Leitz, Germany) by two independent observers. The intensity of the staining was graded according to the following system: -, negative; +, variable; +, weak; ++, moderate; +++, strong. Photographs were taken using colour film Ektachrome 64.

RESULTS

All specimens exhibited a heterogeneity in the morphology of the cells infiltrating the granulomas. Sections from granuloma annulare showed a palisading arrangement of the cells. Generally, a large number of infiltrating cells were encountered in the tissue surrounding the granulomatos mass, e.g. close to sweat glands and blood vessels.

Plasma cells, identified by their morphological appearance, were arranged in a mantle-like fashion at the periphery of the granuloma or in the surrounding tissue. They exhibited an immunoreactivity, which was cytoplasmic for all four cytokines and varied in intensity from weak to moderate for IL-1 alpha and IL-1 beta (Fig. 1a, c), and from weak to strong for IL-6 and TNF-alpha (Fig. 1e, h) (Table I). The strong IL-6 and TNF-alpha labelling was mainly confined to the peripherally orientated plasma cells within the granulomatous mass, while the most centrally located plasma cells had weak or no labelling (Fig. 1f).

The intensity of the IL-6-like immunoreactivity seemed to be correlated with the structure of the chromatin material, thus increasing in intensity the more course the chromatin. TNF-alpha immunolabelling, although mainly cytoplasmic, was also frequently seen to be cell membrane-associated (Fig. 1h).

Epitheloid cells, comprising the majority of the infiltrating cells in the granulomas, showed a variation in their pattern of immunolabelling for the different cytokines, being weak to moderate for IL-1 alpha and IL-1 beta (Fig. 1a, c), and weak to strong for IL-6 and TNF-alpha (Fig. 1d, g). The staining was homogeneous and cytoplasmic for all four cytokines. However, there was no significant difference in the localization of the labelled cells within the granulomatous mass.

Langhans giant cells were occasionally observed at the periphery and/or the centre of the granulomas. IL-6 and TNF-alpha immunoreactivity in the Langhans giant cells was cytoplasmic and homogeneous, and the intensity was almost the same as in the surrounding plasma cells (Fig. 1d, g). Immunolabelling for IL-1 alpha and IL-1 beta was also cytoplasmic but weak to moderate in intensity (Fig. 1e).

Other cells in the infiltrate, with immunolabelling for IL-1 alpha, IL-1 beta, IL-6 and TNF-alpha, were fibroblasts and small...
round cells. The staining observed in these cells was cytoplasmic, but of variable intensity (Fig. 1a, c), while labelling for IL-6 and TNF-alpha was always intense (Fig. 1d, g).

In addition to the labelling in the cellular infiltrate, there was staining of the stroma in the centre of the granulomatous mass from cutaneous leishmaniasis, leprosy and histoplasmosis, especially for IL-6 and TNF-alpha (Fig. 1g, i). Generally, this area contained fewer cells as compared to the periphery of the granuloma, indicating that the granulomas undergo a resolution process.

There was no immunoreactivity in the control sections incubated with PBS instead of primary antibodies. This was also the case for sections treated with antibodies preabsorbed with their respective antigens (Fig. 1b). An exception was the IL-1 beta antiserum, which still showed immunoreactivity even at a high concentration of the antigen.

**DISCUSSION**

Immunohistochemical analysis of IL-1 alpha, IL-1 beta, IL-6 and TNF-alpha cellular infiltrates in different granulomas showed cytoplasmic staining of variable intensity, ranging from weak to strong.

Preabsorption of each cytokine antiserum with the respective antigen resulted in the abolition of immunoreactivity, indicating specificity of staining for IL-1 alpha, IL-6 and TNF-alpha. The IL-1 beta immunoreactivity was not abolished by preabsorption with the antigen. The reason for this is still not clear, but it may be due to insufficient amounts of IL-1 beta in the preabsorption experiment, since large quantities (1 mg) were used in earlier studies (see ref. 18). The staining of IL-6 alpha and IL-1 beta was similar in the present investigation, possibly indicating a cross reaction of the IL-1 beta antiserum with IL-1 alpha.

Studies of cellular interactions which lead to the formation and maintenance of granuloma have been focused on the role of cytokines. Macrophages are activated by antigens that cause cytokine generation and release (8). The findings in the present study are in accordance with this, and with the reported role of mononuclear cells during the development of the granulomatous inflammation, where they control the lesional development via a synthesis of specialized protein communication signals (19).

A role for IL-1 and TNF-alpha in the immune hypersensitivity reaction and in granuloma formation has been suggested (19). IL-1 production by macrophages was maximal during the early development of granuloma and then declined. IL-1 beta was the major species of IL-1 secreted by these macrophages, comprising about 60% of IL-1 activity, while IL-1 alpha constituted only 20%. The remaining IL-1 activity, about 20%, was suggested to be caused by IL-6 (20). Furthermore, there is spontaneous production of IL-1 by mononuclear cells from patients with granulomatous conditions such as leprosy, tuberculosis and sarcoidosis (22), and the ability of IL-1 to induce and/or enhance granuloma formation in vivo has been reported (21-25).

Production of TNF-alpha by macrophages was shown to increase during the late period of granuloma formation (8 to 16 days), suggesting its role in the maintenance of the granuloma. This period coincides with the decrease in IL-1 beta production (20). Since the clinical history of the diseases of our patients was at least 6 months, as in chronic granulomatous inflammation, the above statement is consistent with our results. Notably, locally released TNF-alpha was found to have a central role in the formation and maintenance of BCG granuloma in an autocrine and paracrine manner (26). The precise mechanism by which TNF-alpha contributes to granuloma formation and/or maintenance is still obscure, but it may function by recruitment of additional inflammatory cells, by inducing endothelial cell adherence proteins, granulocyte activation, regulation of fibroblast growth and collagen synthesis (27).

The mantle-like arrangement of plasma cells within the granuloma and the intense immunoreactivity in the peripherally orientated cells and in the surrounding tissue are highly suggestive of B cells being active participants in the granulomatous response. This is further supported by a recent report, which suggested a possible role for B cells in the development and maintenance of granuloma. B cells were found around epithelial cell aggregates in a late lepromatous granulomatous reaction (28).

Our findings of strong IL-6 immunoreactivity in plasma cells suggest that this cytokine may have a role at late stages of the granulomatous response. Furthermore, it has been reported that B cells can produce IL-1, IL-6 and TNF-alpha (29), and that IL-6 is required by activated B cells for their differentiation to antibody-producing cells (30, 31). Thus, the role of B cells and/or IL-6 in granulomatous inflammation, suggested above is possibly achieved by an autocrine or paracrine fashion, since other types of infiltrating cells can produce IL-6 (20-23).

The occurrence of IL-1 alpha, IL-1 beta, IL-6 and TNF-alpha immunoreactivity in the stroma and the finding of less abundantly infiltrating cells in these areas may indicate that these cytokines may be involved in the resolution process of granuloma, perhaps through production by T suppressor cells, which have been found to have a role in this process (32). However, this suggestion contradicts the reported decline in the production of IL-1 and TNF-alpha during the granuloma resolution period (23). Another explanation is that these cytokines are passively released by the degenerating cells within the granuloma during this process.

In conclusion, our findings suggest that IL-6, TNF-alpha and plasma cells are active participants in the maintenance of granuloma. In addition, they are consistent with the reported decreasing role of IL-1 in this phase of the granulomatous reaction. To clarify this speculation we are now studying synchronous granuloma formation in a murine model of cutaneous leishmaniasis.

**ACKNOWLEDGEMENTS**

This study has been supported by a grant from the Welch Foundation. The authors are grateful to Hoffmann-La Roche and Reanal for generously providing rIL-1 alpha and rTNF-alpha, respectively, and to Dr. Stefan Svensson, for the polyclonal antiserum to IL-1 alpha, IL-1 beta and TNF-alpha.

**REFERENCES**


