CC and CXC Chemokines Are Differentially Expressed in Erythema Multiforme In Vivo

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Background: A characteristic feature of erythema multiforme is an acute inflammatory reaction of the skin with an infiltrate largely composed of mononuclear cells around the upper dermal vessels and in the dermal-epidermal interface.

Objective: To determine the composition and localization of leukocyte subsets and corresponding expression of chemokines with chemoattractant properties for lymphocytes and macrophages.

Materials and Methods: Immunohistochemical analysis was performed to localize leukocyte subsets (CD1+, CD3+, CD4+, CD8+, and CD68+). Expression of transcripts and proteins of chemokines (macrophage chemoattractant protein [MCP] 1; macrophage inflammatory protein [MIP] 1α and MIP-1β; regulated on activation, normal T-cell expressed and secreted [RANTES]; growth-related oncogene α; epithelial-derived neutrophil attractant 78; interleukin 8; macrophage interferon-γ inducible gene [Mig]; and interferon-γ inducible protein 10) was determined by in situ hybridization and immunohistochemical analysis.

Setting: Department of Dermatology, University of Würzburg Medical School.

Results: High levels of messenger RNA expression of MCP-1, RANTES, Mig, and interferon-γ inducible protein 10 were detected and localized in the interface zone and subepidermal infiltrate. In contrast, other investigated chemokines (growth-related oncogene α, interleukin 8, epithelial-derived neutrophil attractant 78, I-309, MIP-1α, and MIP-1β) were minimally expressed or absent. Protein expression of MCP-1, RANTES, Mig, and interferon-γ inducible protein 10 was high in the interface zone and low in the subepidermal infiltrate. The messenger RNA expression and protein immunoreactivity patterns overlapped. According to the expression profiles, Mig, interferon-γ inducible protein 10, MCP-1, and RANTES were expressed by basal keratinocytes above and mononuclear cells within the inflammatory foci.

Conclusion: These cytokines are important agents in the cytokine network and contribute to the cell-specific and spatially restricted recruitment of mononuclear cells in the acute inflammation of erythema multiforme lesions.

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Erythema multiforme (EM) is an acute self-limiting mucocutaneous disorder characterized by a pleomorphic eruption. Clinically, the lesions present through papular, vesicular, and target or iris stages.1-3 The classic syndrome is called EM minor and is often preceded by infections with herpes simplex.4 The minor form is a self-limiting, episodic eruption of lesions and presents with a symmetrical distribution and a predilection for the extremities.4,5 Histopathologically, EM is uniform and characterized by a lichenoid tissue reaction with a superficial perivascular lymphohistiocytosis infiltrate and a mononuclear infiltrate at the dermal-epidermal junction.5-7 The inflammation results in basal cell layer injury and subsequent hydropic degeneration of basal cells and keratinocyte necrosis.1-3 As such, EM is considered a prototype of the so-called interface dermatitis and a model lesion for acute inflammation with mononuclear cells.8

Although the cellular composition of the infiltrate in EM has been described in detail,9 the chemotactic factors responsible for the rapid and selective recruitment of mononuclear cells have not been analyzed as yet, to our knowledge.

Chemotaxant cytokines (chemokines) constitute a supergene family and are separated into 4 branches (C, CC, CXC, CX3C), based on a cysteine motif.10,11 The CXC and CC chemokine subfamilies are considered to be the most important.11 Members of the CXC family (alpha-
MATERIALS AND METHODS

SELECTION AND PREPARATION OF SKIN SECTIONS

Fourteen skin biopsy specimens from patients with EM lesions were obtained under local anesthesia. Lesions were biopsied from extremities between days 1 and 4 after onset of the disease. In all patients, the condition was precipitated by a preceding herpes simplex virus infection and was not pretreated before the biopsies were taken. Biopsies from normal skin of healthy volunteers were used as control samples. All specimens were immediately embedded in paraffin or ornithine carbamyl transferase compound (Tissue Tek; Miles Scientific, Naperville, Ill). In the latter case, they were snap-frozen and stored at -80°C until use. Five-µm serial sections were fixed in acetone (10 minutes at 4°C) for immunohistochemical analysis and in 4% paraformaldehyde (20 minutes at room temperature) for in situ hybridization.

ANTIBODIES

For immunohistologic analyses, the following mouse monoclonal antibodies were used at the dilutions indicated: anti-CD1a (1:1000; Coulter Electronics, Krefeld, Germany) recognizing Langerhans cells; anti-CD3 (1:500; Becton, Dickinson and Company, Sunnyvale, Calif); anti-CD4 (1:100; Dako, Hamburg, Germany); anti-CD8 (1:200 and 1:1000, Dako); anti–neutrophil elastase (1:200, Dako) recognizing neutrophils; anti–MIP-1α (1:20; Promega Corporation, Madison, Wis), anti–MCP-1 (1:20; Genzyme Corp, Cambridge, Mass), anti–RANTES (1:20; R&D Systems, Bad Nauheim, Germany), and anti–gro-α (1:20, R&D Systems). The following goat polyclonal antibodies were used: anti–MIP-1β (1:200, R&D Systems), anti–IP-10 (1:300, R&D Systems), and anti–Mig (1:500). Biotin-conjugated sheep anti–mouse immunoglobulin (1:200, Amersham Biosciences, Braunschweig, Germany) and biotin-conjugated mouse anti-goat immunoglobulin (1:500; Jackson Immunoresearch Laboratories, Inc, West Grove, Pa) were used as second-stage reagents.

IMMUNOHISTOLOGIC EXAMINATION

For immunohistologic staining, a 3-step streptavidin-biotin-peroxidase procedure was used as previously described. Briefly, after blocking Fc receptors with 20% sheep serum in phosphate-buffered saline containing 3% skim milk powder and 0.1% Tween 20 (pH adjusted to 7.4) for 30 minutes at room temperature, sections were incubated with the first-step antibody overnight at 4°C, followed by incubation with the respective biotin-conjugated second-step antibody for 1 hour at room temperature and preformed streptavidin-biotin-peroxidase complex (streptABC-peroxidase complex, Dako) for 1 hour at room temperature. Finally, sections were visualized using 3-amino-9-ethylcarbazole dissolved in N,N-dimethylformamide (final concentration 5%) and 0.005% hydrogen peroxide in acetate buffer (50mM at pH 5). For control purposes, the first-step antibody was omitted and replaced by an irrelevant isotype-matched immunoglobulin or control serum. The controls consistently yielded negative results.

IN SITU HYBRIDIZATION

Preparation of sulfur-35-labeled RNA probes was performed as previously described.24 After linearization of plasmid DNA with appropriate restriction enzymes, 35S-labeled sense and antisense probes were obtained by in vitro transcription using SP6, T3, or T7 RNA polymerases (Boehringer Ingelheim GmbH, Mannheim, Germany) together with adenovirus terminal phosphatase, guanosine triphosphate, cytidine triphosphate (Boehringer Ingelheim GmbH) and [35S]uridine triphosphate (Amersham Biosciences) as substrates. After elimination of the original linearized template cDNA with deoxyribonuclease (Pharmacia LKB Biotechnology, Munich, Germany), alkaline hydrolysis of labeled probes was performed for 30 to 50 minutes. After several ethanol precipitation steps, the radioactive riboprobes were adjusted to a specific activity of 2×106 cpm/µL in 0.1M Tris-hydrochloride, pH 7.5, containing 1mM EDTA.

The hybridization procedure was performed as previously described.24 Paraffin-deparaffin-fixed cryostat sections were treated with proteinase K (1 µg/mL, Boehringer Ingelheim GmbH) for 30 minutes at 37°C, refixed in paraffin, acetylated with acetic anhydride in 0.1M triethanolamine (pH 8.0 for 10 minutes), dehydrated, and air dried. The sections were overlaid with 20 µL of hybridization solution (30% formamide, 300mM sodium chloride, 20mM Tris-hydrochloride, pH 8.0, 5mM EDTA, 1× Denhardt solution, 10% dextran sulfate, 100mM dithiothreitol, and 2×106 cpm/µL heat-denatured radioactive probe). After hybridization for 12 to 16 hours, nonhybridized probes were removed by several high stringency washing procedures (50% formamide solution, 2× saline–sodium–citrate buffer [Sigma-Aldrich], and 5mM EDTA at 54-57°C). Nonspecific background RNA was digested with ribonuclease A1 (20 µg/mL) and ribonuclease T1 (1 U/µL, Boehringer Ingelheim GmbH) for 30 minutes at 37°C. For autoradiography, slides were dipped in Kodak type NTB2 emulsion (1:2 in 800mM ammonium acetate [Sigma-Aldrich]) and exposed for 4 weeks at 4°C.

EVALUATION OF SLIDES

For evaluation and documentation of the developed slides, an Axioskop microscope (Carl Zeiss; Oberkochen, Germany) was used. Positive cells were counted with an ocular square grid (Carl Zeiss) at 2 to 4 randomly selected areas of corneal lesions (magnification, ×200 and ×400) and related to the total number of cells. Two to four sections, according to a high or a low number of positively hybridized cells, were evaluated. The mean ± SEM percentage of messenger RNA (mRNA)–expressing cells was determined.

Chemokines (including growth-related oncogene [gro] α, epithelial-derived neutrophil attractant (ENA) 78, and interleukin [IL] 8). These have been shown to attract neutrophils and lymphocytes, the latter at least in vitro. In contrast, the CXC chemokines macrophage interferon-γ inducible gene (Mig) and interferon-γ inducible protein (IP) 10 are selectively attractant for activated T lymphocytes and are both induced by interferon-γ. Chemo-
The chemokines of the CC subgroup (β-chemokines) are composed of macrophage chemoattractant protein (MCP) 1; macrophage inflammatory protein (MIP) 1α and MIP-1β; and regulated on activation, and normal T-cell expressed and secreted (RANTES). These are well known for their chemotactic properties toward macrophages and T-cell subsets.

Several chemokines have been described in psoriasis and point to an important role of these leukocyte attractants in skin inflammation. It is likely that cooperative efforts of a network of chemokines are important for leukocyte recruitment. However, to our knowledge, a comprehensive repertoire of CXC and CC chemokines has not been investigated in an acute inflammatory disease with a predominant mononuclear infiltrate. We, therefore, studied the chemokine profile in 14 EM lesions with an infiltrate consisting of mononuclear leukocytes.

**RESULTS**

For identification and localization of infiltrating leukocyte subsets, immunohistochemical analysis was performed on 14 EM lesions using a panel of mouse monoclonal antibodies that recognize T lymphocytes (anti-CD3), macrophages (anti-CD68), polymorphonuclear cells (anti-neutrophil elastase), and Langerhans cells (anti-CD1). The infiltrate in the examined lesions consisted almost exclusively of mononuclear cells accumulating around upper dermal vessels and along the dermal-epidermal junction. The perivascular infiltrate was mainly lymphocytic, whereas the infiltrate in the dermal-epidermal interface was composed of equal amounts of macrophages and T lymphocytes. The relative percentages of all leukocyte subtypes investigated are summarized in Figure 2. The CD4+ T-lymphocyte subpopulation in the mononuclear infiltrate was greater than the CD8+ T-lymphocyte subpopulation (Figure 1B). In contrast, NE+ neutrophils (Figure 1D) and Langerhans cells were barely detectable.
The immunohistologic results showed that mononuclear cells, namely, lymphocytes and macrophages, are the dominating cell population in the infiltrate. Using in situ hybridization, those chemokines that are suspected to exert a chemoattractant effect on T lymphocytes and macrophages were examined. Using chemokine antisense probes, strong expression of Mig, IP-10, MCP-1, and RANTES was found. These chemokines were highly expressed in the basal dermal-epidermal border and subepidermal infiltrate. The expression of these chemokines was restricted to the area of strong infiltration of lymphocytes and macrophages. In contrast, levels of all other chemokines examined (MIP-1α and MIP-1β, gro-α, 1-309, and ENA-78) were low or barely detectable. The relative percentages of all chemokines expressing mRNA in cells were quantified and summarized in Figure 3. Cell-associated in situ hybridization signals were barely detectable in control sections of normal skin hybridized with antisense probes.

The most conspicuous finding in this study was the intense chemokine expression in the interface zone. Macrophage chemoattractant protein 1, RANTES, Mig, and IP-10 were highly expressed by basal keratinocytes and adjacent leukocytes (Figure 4). Macrophage chemoattractant protein 1, RANTES, and Mig, but not IP-10, showed higher expression levels in lesions with larger infiltrates compared with lesions with smaller infiltrates. Expression of all other examined chemokines (MIP-1α and MIP-1β, gro-α, ENA-78, and IL-8) was low and confined to leukocytes localized below the epidermis and the subepidermal infiltrate (data not shown).

If the composition of inflammatory cells is affected by selective recruitment and trafficking through chemokines, expression of chemokines with lymphocyte and macrophage chemoattractant properties should dominate EM lesions. To confirm this hypothesis, the number of cells expressing chemokines with lymphocyte chemoattractant properties (gro-α, IL-8, Mig, IP-10, MCP-1, MIP-1α and MIP-1β, and RANTES) was compared with the number of cells that express chemokines with monocyte or macrophage chemoattractant properties (MCP-1, MIP-1α and MIP-1β, and RANTES) or neutrophil chemoattractant properties (gro-α, ENA-78, and IL-8). As shown in Figure 3, more cells expressed lymphocyte chemoattractant chemokines (Mig, IP-10, MCP-1, and RANTES) than monocyte or macrophage chemoattractant chemokines (MCP-1 and RANTES), whereas expression of neutrophil chemoattractant chemokines was low. Therefore, the differential chemokine expression profile with a slight dominance of lymphocyte chemoattractant chemokines reflected the composition of the infiltrates, showing a preponderance of CD3+ lymphocytes compared with CD68+ macrophages. The low expression levels of the neutrophil chemoattractant chemokines gro-α, ENA-78, and IL-8 also correlate with a small presence of neutrophils in EM.

In addition to the quantification of chemokine expression, we studied the microanatomic localization of chemokine expression and found that MCP-1 transcripts were detected in basal keratinocytes and subepidermal cells (Figure 4A). In contrast to MCP-1, RANTES mRNA expression was greater in the interface zone than in the subepidermal infiltrate (Figure 4C and D). Epidermal expression of RANTES mRNA was mainly detected in basal keratinocytes that were associated with a subepidermal infiltrate (Figure 4C and D). Interferon-γ inducible protein 10 mRNA expression dominated in the basal epidermis (Figure 4F). The basal cell layer and 2 to 4 suprabasal cell layers of the epidermis expressed IP-10 mRNA (Figure 4F). In contrast, Mig mRNA was strongly expressed in the subepidermal infiltrate (Figure 4G and H).

To exclude the possibility that chemokine mRNA expression is not paralleled by translation into protein, sections were labeled with monoclonal antibodies or polyclonal antiserum against MCP-1, MIP-1α and MIP-1β, RANTES, Mig, and IP-10. Strong chemokine immunoreactivity, localized to mononuclear cells, and particularly to basal keratinocytes, was detected for MCP-1 (Figure 4B) and RANTES (Figure 4E), whereas only single cells were positively labeled for MIP-1α and MIP-1β (data not shown). Nonspecific background labeling, however, was obvious and focally intense in the epidermal compartment. The mRNA expression and protein immunoreactivity patterns in the dermis mainly overlapped, indicating translation of mRNA into protein in vivo.

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**COMMENT**

The infiltration of mononuclear cells around superficial blood vessels and in the dermal-epidermal junction is a hallmark of EM lesions. In this study, we attempted to elucidate the expression of CC and CXC chemokines involved in the mechanisms responsible for the infiltrate, mainly composed of lymphocytes and macrophages.

This study demonstrates the differential expression of chemokines in EM lesions. As shown in the "Results" section, the chemokines MCP-1, RANTES, Mig, and IP-10 were highly expressed, whereas expression levels of gro-α, ENA-78, and MIP-1α and MIP-1β were low or barely detectable. In addition, the chemokine expression patterns differed. While IP-10 and RANTES expression was concentrated in the interface zone, Mig and
Figure 4. Macrophage chemoattractant protein (MCP-1) messenger RNA (mRNA) is highly expressed in the basal epidermis and subepidermal infiltrate (A) and correlates with MCP-1–specific immunoreactivity localized in the interface zone and the subepidermal infiltrate (B). Regulated on activation, normal T-cell expressed and secreted (RANTES) mRNA is localized in the perivascular infiltrate and lesional keratinocytes (C, D) and correlates with RANTES immunoreactivity, which is mainly restricted to basal epidermis (E). Interferon-γ inducible protein (IP-10) mRNA is highly expressed in the basal epidermis (F). The macrophage interferon-γ inducible gene (Mig) is mainly expressed in the subepidermal infiltrate (G, H) (in situ hybridization with [35S]uridine triphosphate-labeled antisense probes [A, C, D, F-H]; bright-field illumination [A-C, E-G]; dark-field illumination [D, H]; and 3-step streptavidin-biotin-peroxidase method [B, E]) (panels A-H stained with Papanicolaou). Bar is 50 µm.
MCP-1 mRNA was also highly expressed in the subepidermal infiltrate. This chemokine profile—dominance of lymphocyte attractant chemokines and high expression at the dermal-epidermal junction—may explain the microanatomic features and composition of infiltrates in lesions of the interface dermatitis EM, with strong infiltration particularly in the dermal-epidermal junction. Taken together, the composition of the cellular infiltrate (more lymphocytes than macrophages) is well reflected by the chemokine profile. There is a higher number of lymphocyte attractant chemokines (Mig, IP-10, MCP-1, and RANTES) with higher expression levels, compared with monocyte or macrophage attractant chemokines (MCP-1 and RANTES). In all examined lesions, the size of the infiltrate correlated with the amount of mRNA signals of the investigated chemokines. The low expression levels of the neutrophil attractant chemokines (gro-α, ENA-78, and IL-8) are also well reflected by the low number of neutrophils in EM lesions.

Several studies in recent years have shown that chemokines have overlapping in vitro functions. They also have diminished the hope that one of them was dominant expressed in vivo and that a specific receptor blockade could be therapeutically beneficial. In EM and other skin diseases, chemokines are expressed differentially. In the interface dermatitis lichen planus, other skin diseases, chemokines have overlapping in vitro functions. They also induce the cytotoxic response of T lymphocytes. These recently proliferated keratinocytes would lack antigens that might induce the cytotoxic response of T lymphocytes. These results and the results of the aforementioned chemokine studies suggest that the effects of chemokines in vivo probably lie on a continuum between benefit and harm, depending on their local concentrations and the state of the cytokine network. Selective receptor blockade of highly expressed chemokines in inflamed eye tissue could then be explored as a reasonable strategy to inhibit the recruitment of leukocytes.

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