Differential and Sequential Expression of Multiple Chemokines during Elicitation of Allergic Contact Hypersensitivity

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Regulation of chemokine-mediated leukocyte migration within inflammatory tissues is a complex event that cannot be mimicked and analyzed *in vitro*. We therefore investigated the role of macrophage- and T-lymphocyte-specific chemoattractants involved in the positioning of immune effector cells during the elicitation phase of contact hypersensitivity, a prototype of a T-lymphocyte-mediated immune reaction. Serial sections of skin biopsies obtained from sensitized individuals at distinct time intervals after epicutaneous application of allergens were hybridized with anti-sense probes of a large panel of chemokines or immunohistologically labeled with leukocyte-specific antibodies. Multifocal expression of monocyte chemoattractant protein-1 (MCP-1) was already detected after 6 hours in basal keratinocytes clearly preceding the infiltration of monocytes and T cells. Increasing basal expression of MCP-1 and, in addition, of regulated upon activation, normal T-cell expressed and secreted (RANTES) after 12 hours was accompanied by dermal expression of MCP-1, macrophage-derived chemoattractant (MDC), and RANTES and paralleled by infiltration of mononuclear cells into dermis and epidermis. Expression of the T-lymphocyte-specific chemokines IP-10 and MIG in epidermis and dermis and of MDC, pulmonary and activation-regulated chemokine (PARC), and thymus and activation-regulated chemokine (TARC) exclusively in the dermis started after 12 hours reaching maximum levels at 72 hours and was associated with infiltration of T cells into the epidermal compartment. Our data provide evidence that migrating effector cells encounter multiple chemoattractant signals in a complex spatial and temporal pattern. In particular, keratinocytes contribute to the vigorous immigration by sequential expression of MCP-1, RANTES, and interferon-inducible protein-10 (IP-10) monokine induced by gamma interferon (MIG), indicating that chemokine-mediated nonimmunological mechanisms precede and corroborate

antigen-specific mechanisms during elicitation of contact hypersensitivity. (Am J Pathol 2001, 158:431–440)

Hapten-induced contact hypersensitivity (CHS) is a highly frequent, often occupationally related human skin disorder in industrialized countries with an enormous sociomedical impact.¹ Moreover, CHS is considered as a standard model for an antigen-specific, T-lymphocyte-mediated immune response.² This concept still holds true for the clinically nonapparent sensitization phase of CHS with antigen-processing and presentation by Langerhans cells and a consecutive T cell stimulation in the draining lymph node. However, there is increasing evidence that during the clinically visible and physically disturbing elicitation phase of CHS nonspecific hapten-induced proinflammatory effects precede or parallel the antigen-specific effect and are a *conditio sine qua non* for the vigorous inflammatory reactions.²

In a mouse CHS model Grabbe and colleagues³ demonstrated that nonspecific effects of epicutaneously applied haptens contribute to the elicitation of CHS. Proinflammatory irritative rather than antigen-specific properties of the haptens are furthermore responsible for the strict concentration-dependence of the effector phase of CHS. Therefore, it is tempting to speculate that such irritative properties of haptens promote inflammatory skin reactions via induction of proinflammatory cytokines, adhesion molecules, and chemoattractants. Accordingly, some contact allergens such as urushiol, the relevant hapten in poison ivy, and nickel sulfate have been demonstrated to directly induce inflammatory activation of keratinocytes resulting in expression of ICAM-1, interleukin (IL)-8, and/or tumor necrosis factor (TNF)- α .^{4–6} In recent years, in particular chemokines have emerged as potent stimulators of effector cell accumulation and activation and are likely candidates to mediate leukocyte recruitment during elicitation of CHS. Since the description of IL-8 more than a decade ago, the supergene

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family of chemokines has increased enormously and comprises a set of more than 30 different species.⁷⁻⁹ For many of them, the detailed understanding of their *in vivo* role is fragmentary or even lacking. This is mainly because of the limited availability of *in situ* data obtained under pathological conditions. The individual role of chemokines during CHS has been studied only for single species (eg, IP-10, MCP-1, Eotaxin) and mainly in the murine system.¹⁰⁻¹³ Because mouse skin exhibits quite a different morphology and physiology as compared to human skin (eg, only few keratinocyte layers, high abundance of hair follicles) and because chemokine homologues for both the human and murine system are not always established, inflammatory disorders such as CHS reactions should be studied, despite experimental restrictions, in the human system itself. As most chemokines are regarded to be redundant in their action on target cells, promiscuous in receptor usage, and produced by both resident and passenger cells (leukocyte subtypes) of the skin,¹⁴ understanding of the inflammatory response in CHS requires the *in vivo* study of an extended set of chemokines. Furthermore, diapedesis and migration from the dermal into the epidermal compartment may be regulated by a sequential and spatial different set of chemokines as recently demonstrated for other inflammatory conditions of the skin.^{15,16} In particular, the elegant *in vitro* studies of neutrophil locomotion through complex fields of overlapping chemoattractant gradients by Foxman and colleagues¹⁷ have strengthened the still unproven concept that the spatial guidance of cells in tissue may not be a linear event of a single chemokine gradient but rather requires a complex network of sequential and combinatorial chemoattractant effects. To prove this concept of effector cell recruitment and positioning *in vivo* we applied *in situ* hybridization to study a large panel of radiolabeled chemokine anti-sense probes on serial tissue sections of biopsies obtained at various time intervals after allergen exposure to the skin of sensitized volunteers. We particularly focused our attention on chemokines that are chemoattractive for monocytes and/or lymphocytes such as MCP-1, MCP-3, macrophage inflammatory protein-1 α (MIP-1 α) and β , RANTES, MDC, I309, IP-10, MIG, liver and activation regulated chemokine (LARC), (synonymous with Exodus-1/MIP-3 α), PARC (synonymous with DC-CK1/MIP4), TARC, lymphotactin, stromal cell-derived factor-1 (SDF-1 α) and β , and interferon-inducible T cell alpha chemoattractant (I-TAC). In addition, we studied expression of IL-8, growth-related oncogene α (Gro α)/MGSA, granulocyte chemoattractant protein-2 (GCP-2), and endothelial neutrophil-attracting protein 78 (ENA-78) that primarily attract neutrophils as well as of hemofiltrate C-C chemokine (HCC-1). The microanatomical location of chemokine expression and the longitudinal profile was semiquantitatively evaluated and compared with the infiltration pattern of leukocyte subtypes. We demonstrate that during elicitation of CHS a highly diversified repertoire of chemokines is expressed at distinct sites of the skin that spatially and temporally correlates with the recruitment of macrophages and T cells.

Materials and Methods

Study Participants

Thirteen patients referred to the Allergy Section of the Department of Dermatology, University of Würzburg, for evaluation of contact allergies by patch testing were included in this study after having given informed written consent. The study was approved by the Ethics committee at the University of Würzburg. Separate epicutaneous patch tests were performed on the volar forearm using occlusive Finn Chambers (Hermal, Reinbek, Germany). Standard concentrations of allergens prepared in soft paraffin (Hermal) were used to reproduce CHS reactions. All patch-test chambers were removed at the time of biopsy or after a maximum of 48 hours of contact. After local anesthesia with mepivacaine, 5-mm-punch skin biopsies (four to five biopsies from each individual) were taken from the patch-test sites before and 6, 12, 24, 48, 72, and/or 96 hours after allergen application. Evaluation of quantitative data is based on results obtained from at least four biopsies per time point. CHS reactions were elicited in five patients by 5% nickel sulfate; in three patients by 20% colophony; and in one patient by each isocillin, 2% nystatin, 0.25% sodium thiosulfatoaurate, 1% paraphenylenediamine, or 20% neomycin sulfate, respectively. Tissue samples were embedded in OCT compound (Tissue Tek, Diatec, Nürnberg, Germany) immediately after punch biopsy, frozen, and stored at -80°C . Cryostat sections (5 μm) were prepared on gelatin-coated slides (Merck, Darmstadt, Germany) for immunohistology and on poly-L-lysine-coated slides (Sigma, Deisenhofen, Germany) for *in situ* hybridization. After air-drying, sections were fixed in acetone (10 minutes, 4°C) for immunohistology or, for *in situ* hybridization, in 4% paraformaldehyde/phosphate-buffered saline (PBS) (20 minutes, room temperature; Sigma). Alternatively, specimens were fixed in 4% formaldehyde and embedded in paraffin wax for a superior microanatomical preservation.

Immunohistology

A three-step streptavidin-biotin-complex (StreptAB-Complex)/peroxidase method was used as previously described.¹⁸ The following monoclonal antibodies (mAbs) were used: anti-CD3 (at 1:500; BD, Sunnyvale, CA), reacting with the T cell receptor-associated CD3 antigen; anti-CD4, anti-CD8 (both at 1:200; BD); anti-HLA-DR (at 1:500; BD); anti-CD68 (clone KP-1, at 1:1,000; DAKO, Copenhagen, Denmark), recognizing macrophages; anti-neutrophil elastase (at 1:200; DAKO), specifically labeling neutrophils; anti-CXCR1 (clone 5A12 at 1:1,000; PharMingen, Hamburg, Germany), anti-CXCR2 (clone 6C6 at 1:1,000, PharMingen) and anti-CXCR3 (clone 44716.111 at 1:500; R&D Systems, Wiesbaden, Germany) against human chemokine receptors. After blocking Fc receptors with 20% heat-inactivated sheep serum in PBS, sections were incubated with primary mAb at 4°C overnight, followed by incubation with biotin-conjugated sheep anti-mouse IgG (Amersham Pharmacia Biotech,

Freiburg, Germany) at 1:200 and preformed StreptAB-Complex/Peroxidase (DAKO) at room temperature for 1 hour. Sections were washed between each step and the reaction cascade was visualized by incubation with 3-amino-9-ethylcarbazole (Sigma) as substrate. For control purposes the primary mAb was replaced by isotype-matched IgG of nonrelevant specificity.

In Situ Hybridization

Preparation of ^{35}S -Labeled RNA Probes

cDNA probes were provided by J. M. Farber (NIH, Bethesda, MD), MIG; T. Yoshimura (NCI, Frederick, MA), MCP-1 and Eotaxin-1; Genetics Institute (Cambridge, MA), MIP-1 α ; T. Schall (DNAX Research Institute, Palo Alto, CA), MIP-1 β and RANTES; J. A. Hedrick (DNAX Research Institute), lymphotactin; A. Anisowicz (Dana Farber Cancer Institute, Boston, MA), GRO α ; C. Weissmann (University of Zürich, Zürich, Switzerland), IL-8; C. Müller (University of Bern, Bern, Switzerland), MCP-3; R. Kulke (University of Kiel, Kiel, Germany), ENA-78; M. Krangel (Duke University Medical Center, Durham, NC), I309; W. G. Forssmann (Lower Saxony Institute for Peptide Research, Hannover, Germany), HCC-1; J. B. Smith (UCLA Medical Center, Los Angeles, CA), GCP-2; H. Nomiya (Kumamoto University, Kumamoto, Japan), PARC; O. Yoshie (Shionogi Institute for Medical Science, Osaka, Japan), LARC; R. Godiska and P. Gray (ICOS, Bothell, WA), MDC; and T. Honjo (Kyoto University Faculty of Medicine, Kyoto, Japan), SDF α and β .

IP-10, TARC, and I-TAC were cloned by polymerase chain reaction using the following primers: IP-10, 5'-CGC-AAG-CTT-CGG-GAG-ACA-TTC-CTC-AAT-TGC-3' and 5'-CGC-GGA-TCC-AGG-AGA-TCT-TTT-AGA-CAT-TTC-3' (with *Hind*III and *Bam*HI restriction sites); TARC, 5'-ATG-GCC-CCA-CTG-AAG-ATG-3' and TCA-AGA-CCT-CTC-AAG-GCT-3'; and I-TAC, 5'-CGG-GAT-CCC-GAT-GAG-TGT-GAA-GGG-CAT-3' and 5'-CCG-CTC-GAG-CGG-TTA-AAA-ATT-CTT-TCT-TTC-3'. For polymerase chain reaction amplification of TARC, PHA-stimulated peripheral blood mononuclear cells were used whereas I-TAC and IP-10 were generated from interferon (IFN)- γ -stimulated buffy-coat monocytes. Total RNA was then isolated and cDNA prepared using the primers described above according to standard protocols. *In vitro* transcription of sense and anti-sense probes was performed as described earlier.^{15,16} Radiolabeled probes were obtained by incubation of linearized plasmids with either T7, T3, or SP6 RNA polymerases and ATP, GTP, CTP (all obtained from Roche Molecular Biochemicals, Mannheim, Germany) and ^{35}S -UTP (Amersham Pharmacia Biotech) as substrates. The original template cDNA was eliminated by DNase treatment and protein components were removed by several phenol extraction steps. To facilitate the intracellular accessibility of labeled probes, alkaline hydrolysis was performed to get an average length of 50 to 150 bp. The radioactive probes were adjusted to a specific activity of 2×10^6 cpm/ μl in 0.01 mol/L Tris-HCl, pH 7.5, containing 1 mmol/L ethylenediaminetetraacetic acid.

Hybridization Procedure

In situ hybridization was performed as previously described.^{15,16} Cryostat sections were fixed in 4% paraformaldehyde and treated with 1 $\mu\text{g}/\text{ml}$ proteinase K (Roche Molecular Biochemicals). Sections were acetylated with acetic anhydride in 0.1 mol/L triethanolamine (pH 8.0, 10 minutes), dehydrated in alcohol, and air-dried. Sections were then overlaid with 20 μl of hybridization solution (50% formamide, 300 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8.0, 5 mmol/L ethylenediaminetetraacetic acid, 1 $\times$ Denhardt's solution, 10% dextran sulfate, 100 mmol/L dithiothreitol, and 2×10^5 cpm/ μl heat-denatured radioactive probe). All sense (control) and anti-sense probes were hybridized to at least three sections from the same biopsy. RNase treatment was used as a further control and consistently abrogated specific hybridization signals. After hybridization, sections were washed with a solution containing 50% formamide, 2 $\times$ standard saline citrate (Sigma), and 5 mmol/L ethylenediaminetetraacetic acid at high stringency (54 to 57°C). To remove nonhybridized probes, slides were treated with RNase A (20 $\mu\text{l}/\text{ml}$) and RNase T1 (1 U/ml; Roche Molecular Biochemicals) for 30 minutes at 37°C. To visualize the hybridization reaction, slides were dipped in NTB solution (Kodak) and exposed for 1 to 4 weeks at 4°C.

Assessment of Leukocyte Subsets and Chemokine mRNA-Expressing Cells

Slides processed for *in situ* hybridization or immunohistochemistry were evaluated and documented with an Axiophot microscope (Zeiss, Oberkochen, Germany) equipped with interference contrast, epipolarization, and dark field illumination. For quantification of leukocyte subsets, positive cells in the dermis were counted using an

Figure 1. Time course of leukocyte recruitment into the dermis during elicitation of CHS. Inflammatory cells were identified by immunostaining with mAbs against CD3 (T lymphocytes, filled circle), CD68 (macrophages, open circle) and neutrophil elastase (NE) (neutrophils, filled square). CHS was elicited as described in Materials and Methods and biopsies obtained at the time intervals indicated. Results are presented as percentage $\pm$ SEM of stained cells related to the total number of cells of at least three randomly selected dermal areas. For each time point, four to seven sections were evaluated (for details, see Materials and Methods).

Figure 2. MCP-1 expression during elicitation of CHS. Biopsies obtained at 6 hours (A), 12 hours (B), 24 hours (C), and 48 hours (D) after epicutaneous application of hapten were processed for *in situ* hybridization using a MCP-1 anti-sense probe as described in Materials and Methods. The arrows point to focal expression of MCP-1 mRNA in the basal epidermal layer at 6 hours (A). At 12 hours, there is a nearly continuous expression and at 24 and 48 hours a strong continuous expression in the basal layer. At 24 and 48 hours, strong MCP-1 message is also detectable in the dermal compartment in a perivascular distribution at sites of leukocyte accumulation (C and D). Dark-field illumination. Objective, $\times 10$.

ocular grid. They were related to the total number of cells in dermal areas whereas resident epidermal cells and cells infiltrating the epidermis were not being considered. Biopsy specimens from four to seven individual CHS reactions for each time point were examined. At least three randomly selected fields were evaluated in each case. Data are expressed as percentage of positive cells $\pm$ SEM. In addition, if applicable, the site of preferential expression was photographically documented.

Results

Elicitation of CHS Is Accompanied by Spatially and Temporally Changing Patterns of Infiltrating Inflammatory Cells

In a first series of experiments, sections of biopsies taken at distinct time intervals (0, 6, 12, 24, 48, 72, and 96 hours) after epicutaneous application of contact allergens were histologically evaluated. After 6 hours of allergen application, the histological picture still resembled that of normal skin whereas after 12 hours a slight intercellular edema in the epidermis and some CD3+ T lymphocytes and CD68+ macrophages scattered around superficial and deeper dermal vessels as well as single macrophages immediately below the epidermis could be observed (data not shown). After 24 hours, multifocal infiltration of T cells into the epidermis with concomitant formation of epidermal microvesicles occurred whereas monocytes/macrophages almost exclusively resided in the subepidermal region. Later, at 48 to 72 hours, mononuclear cells increasingly infiltrated the upper dermal compartment and pronounced epidermal edema with vesiculation was visible. At this stage, neutrophils were detectable within the edematous or blistered epidermis. Despite a variable degree of infiltration and vesiculation between biopsies obtained from different patients at the

Figure 3. Expression of macrophage-attracting chemokines MCP-1, RANTES, and MIP-1 α 48 hours after elicitation of CHS. *In situ* hybridization with anti-sense probes against MCP-1 (A and B), RANTES (C and D), and MIP-1 α (E and F) was performed on serial skin sections to compare relative intensity and localization of chemokine message. Both MCP-1 and RANTES are preferentially expressed in lesional basal keratinocytes with few positive cells in the upper dermis. MIP-1 α mRNA+ cells are sparse. Illumination: bright field (A, C, and E), dark field (B, D, and F). Objective, $\times 20$.

same time point after allergen exposure, the relative composition of infiltrates was comparable with a clear predominance of CD3+ T cells and CD68+ macrophages reaching maximum densities at 48 to 72 hours. Composition and time course of infiltrate formation during elicitation of CHS are summarized in Figure 1.

Figure 4. Quantification of cells expressing MCP-1 (filled circle), RANTES (open circle), MIP-1 α (filled square) and MIP-1 β (open square) mRNA after elicitation of CHS. *In situ* hybridization for these chemokines was performed as described in Materials and Methods. Results are expressed as mean ($\pm$ SEM) percentage of specific mRNA-expressing dermal cells per counting field of four to seven sections at each time point until 96 hours after elicitation of CHS.

MCP-1 and RANTES Are the Dominant Monocyte/Macrophage Attractants Expressed during the Elicitation Phase of CHS

To assess the time course and the expression patterns of chemokines attracting monocytes and macrophages, serial sections of biopsies taken before or 6, 12, 24, 48, 72, and 96 hours after allergen exposure were hybridized with anti-sense probes of I309, MCP-1, MCP-3, RANTES, MIP-1 α , and MIP-1 β . In parallel, serial sections were studied for localization of leukocyte subsets.

Before application of allergens (ie, at 0 hours) significant mRNA expression of the chemokines studied was not detectable. After 6 hours of allergen exposure, multifocal expression of MCP-1 was already visible in single basal keratinocytes as shown in Figure 2A. The observation that MCP-1 was the first chemokine found to be up-regulated after elicitation of CHS was made regardless of the kind of allergen applied. Notably, at this early time point the dermal compartment still appeared to be unchanged and an increased number of inflammatory cells could not be detected. At 12 hours, MCP-1 mRNA expression was further up-regulated with an even and strong expression in the basal epidermal layer and a weak expression in single vascular and perivascular cells (Figure 2B) accompanied by a rather scarce perivascular infiltrate (data not shown). MCP-1 mRNA expression further increased up to 48 hours as demonstrated in Figure 2, C and D, and declined after 72 hours. At these time points, despite a massive expression in basal keratinocytes overall expression of MCP-1 was higher in the dermal compartment because of a larger number of strong MCP-1 mRNA expressing cells (Figure 2, C and D). Expression of RANTES was first detected between 12 and 24 hours in keratinocytes of the basal layer in a pattern resembling that of MCP-1. Later, RANTES expres-

sion in the basal layer further increased and was observed in mononuclear cells immediately below the epidermis (Figure 3, C and D). Expression of most other monocyte-attractant chemokines was weak (MIP-1 α and MIP-1 β ; Figure 3, E and F) or undetectable (MCP-3, I309; data not shown). A semiquantitative evaluation of chemokine mRNA-expressing cells is shown in Figure 4. In addition to the differential expression patterns, the specificity of hybridization was further confirmed using sense probes that did not result in cell-associated hybridization signals (data not shown).

Increasing expression of monocyte-attractant chemokines was paralleled by an accumulation of monocytes/macrophages as evaluated on serial sections by immunohistochemistry. The latter was found to be restricted to sites of chemokine expression (Figure 5; A, B, and C). Accordingly, monocytes/macrophages were only rarely detected in the epidermis beyond the basal layer.

The Lymphocyte-Attractant Chemokines IP-10, MIG, MCP-1, MDC, RANTES, PARC, and TARC Are Highly and Differentially Expressed during Elicitation of CHS

Because CHS is considered as a prototype of a T-cell-mediated inflammatory reaction, it was mandatory to assess the expression patterns of chemokines exhibiting lymphocyte-attractant properties. Serial skin sections were hybridized with anti-sense probes of IP-10, MIG, MDC, RANTES, LARC (Exodus-1), PARC (DC-CK1, MIP-4), TARC, MIP-1 α and - β , MCP-1 and -3, lymphotactin, I-TAC, HCC-1, IL-8, Gro α , and SDF α and - β . Among all chemokines studied MIG- and IP-10-specific probes showed the highest levels of cell-associated signals. Expression of these started between 12 and 24 hours after allergen application and reached maximal levels after 72 hours (Figure 6). Notably, both chemokines were preferentially expressed at identical sites of the epidermis and, unlike MCP-1, could also be observed at suprabasal layers of the epidermis after 48 hours (Figure 6). In serial sections, epidermal MCP-1 mRNA expression was always restricted to the basal layer. Increasing expression of IP-10 and particularly of MIG was additionally detected in the subepidermal region and co-localized expression of both chemokines correlated with dense infiltration of CD3+ and CXCR3+ lymphocytes in the dermis and exocytosis into the epidermis (Figure 5; D, E, and F; and data not shown). MCP-1 and RANTES, which attract both monocytes and lymphocytes, exhibited a similar expression pattern, albeit with another time course as described for IP-10 and MIG and were not detected to be expressed in suprabasal layers. In particular, MCP-1 expression started much earlier than that of IP-10 and MIG. A quantitative evaluation of IP-10 and MIG mRNA expression is given in Figure 7.

In contrast to these chemokines, PARC and TARC were expressed in perivascular clusters at sites of leukocyte accumulation exclusively in the upper dermis (Figure 8). MDC mRNA was first found to be moderately

Figure 5. Spatial and temporal correlation between chemokine expression and recruitment of corresponding target cells. Sections obtained 24 hours after elicitation of CHS were hybridized with radioactively labeled anti-sense probes of MCP-1 (**A** and **B**) and MIG (**D** and **E**). Corresponding serial sections were immunohistologically labeled with macrophage (anti-CD68) (**C**) or T-lymphocyte-specific (anti-CD3) (**F**) mAbs as described in Materials and Methods. Expression of MCP-1 (**A** and **B**) and MIG (**D** and **E**) is overlapping with areas of macrophage (**C**) and T-lymphocyte (**F**) infiltration, respectively. Illumination: bright field (**A**, **C**, **D**, and **F**), dark field (**B** and **E**). Objective, $\times 10$.

expressed by perivascular mononuclear cells around superficial vessels at 12 hours (data not shown). Maximal expression levels after 48 to 72 hours were frequently detected in areas of strong leukocyte infiltration and were sometimes much stronger than expression of MCP-1 (Figure 9). Single mononuclear cells directly below the epidermis or intermingled between basal keratinocytes and thus identified as monocytes/macrophages also ex-

pressed MDC message; however, as opposed to MCP-1, MDC mRNA has never been detected in epidermal keratinocytes (Figure 9, C and D). The time course and quantity of MDC, LARC, PARC, and TARC mRNA expression is summarized in Figure 10. All other chemokines studied were only weakly expressed or not detectable (data not shown). IL-8 and GRO α which, besides their role as important neutrophil attractants, have also been implicated in T cell trafficking,^{19,20} were found to be weakly expressed in the epidermis (IL-8) or both the dermis and epidermis (GRO α ; data not shown). Expression of IL-8 and GRO α was multifocal and especially

Figure 6. Pronounced expression of IP-10, MIG, and MCP-1 mRNA 48 hours after elicitation of CHS. Serial skin sections were processed for *in situ* hybridization using ³⁵S-UTP-labeled anti-sense probes of IP-10 (**A** and **B**), MIG (**C** and **D**), and MCP-1 (**E** and **F**). Simultaneous IP-10, MIG, and MCP-1 expression is co-localized in the basal epidermal layer; notably, IP-10 and MIG mRNA is also strongly expressed by suprabasal cells. Illumination: bright field (**A**, **C**, and **E**), dark field (**B**, **D**, and **F**). Objective, $\times 10$.

Figure 7. Quantification of cells expressing IP-10 and MIG mRNA during elicitation of CHS. *In situ* hybridization for these chemokines was performed as described in Materials and Methods. Results are expressed as mean percentage of specific mRNA-expressing cells per counting field of four to seven sections ($\pm$ SEM) at each time point until 96 hours after allergen exposure.

Figure 8. Differential expression of chemokines MCP-1, MIG, PARC, and TARC 48 hours after elicitation of CHS. *In situ* hybridization was performed with ³⁵S-UTP-labeled anti-sense probes of MCP-1 (A and B), MIG (C and D), PARC (E and F), and TARC (G and H) using serial sections. MIG mRNA signals are focally concentrated in the epidermis (C, arrows) whereas PARC and TARC messages are exclusively expressed below in the dermal compartment. Illumination: bright field (A, C, E, and G), dark field (B, D, F, and H). Objective, $\times 10$.

seen in areas of epidermal edema and blister formation that were infiltrated by single neutrophil elastase+, CXCR1+, and CXCR2+ cells, ie, neutrophils (data not shown). These observations suggest that, during elicitation of CHS, both CXC chemokines influence neutrophil

Figure 9. Expression of MCP-1 and MDC 72 hours after elicitation of CHS. *In situ* hybridization was performed with anti-sense probes for MCP-1 (A and B) and MDC (C and D) on serial sections. In contrast to MCP-1, MDC is mainly expressed by inflammatory cells of the dermis and by a few cells invading the epidermis. Illumination: bright field (A and C), dark field (B and D). Objective, $\times 10$.

Figure 10. Quantification of cells expressing LARC, PARC, TARC, MDC mRNA during the course of CHS. *In situ* hybridization for these chemokines was performed as described in Materials and Methods. Results are expressed as mean percentage of specific mRNA-expressing dermal cells per counting field of four to seven sections ($\pm$ SEM) at each time point until 96 hours after elicitation of CHS.

recruitment rather than T cell migration. Message of neutrophil-attracting chemokines ENA-78 and GCP-2 could not be detected at all (data not shown).

A semiquantitative evaluation of chemokine-expressing cells is given in Figure 10. In summary, during elicitation of CHS the chemokines MIG, IP-10, RANTES, and MCP-1 are strongly expressed in the epidermis and, to a lesser extent, in the dermis. Furthermore, perivascular expression of MDC, LARC, and PARC can be observed that is accompanied by infiltration of lymphocytes.

Discussion

Understanding CHS as the classical model of a T-cell-dominated inflammatory reaction requires a profound knowledge of the mechanisms responsible for leukocyte recruitment to the skin. Trafficking of both monocytes and lymphocytes in CHS is a multistep event²¹ with migration through different skin compartments (blood vessel—dermis—epidermis). We hypothesized that for such a complex navigation expression of single adhesion molecules and chemokines as previously assumed^{4,22,23} is not sufficient. We now demonstrate that recruitment of mononuclear cells during elicitation of CHS is mirrored by spatially and temporally distinct expression patterns of multiple monocyte- and lymphocyte-attractant chemokines in both the dermis and epidermis. MCP-1 is identified as the first chemokine appearing that is expressed in the basal epidermal layer as early as 6 hours after allergen contact thus clearly preceding infiltration by monocytes and lymphocytes. At 12 hours, epidermal expression of MCP-1 further increased and also became detectable in vascular and perivascular cells. It was accompanied by epidermal and, to a lesser extent, dermal expression of RANTES and exclusive dermal expression of MDC. At 24 hours, multifocal epidermal expression of

IP-10 and MIG and dermal expression of TARC and PARC accompanied the increasing expression of MCP-1, RANTES, and MDC. It coincided with strong infiltration of monocytes and lymphocytes. All other chemokines investigated were only minimally expressed (eg, MIP-1 α and - β , GRO α , IL-8, and lymphotactin) or undetectable (I309, LARC, Eotaxin-1, I-TAC, GCP-2, ENA-78). Our study which we believe is the first that analyzed the complexity of chemokine action *in vivo* indicates that a restricted and timely changing set of chemokines governs the migration of monocytes and lymphocytes during evolving CHS in human skin. Notably, with the exception of IP-10, MIG, PARC, and TARC as selective T cell attractants, most other chemokines detected in CHS (MCP-1, RANTES, MDC) are more or less chemoattractant for both monocytes and lymphocytes but not for neutrophils.^{7,9} This may explain the predominance of mononuclear cells in CHS and the simultaneous immigration of lymphocytes and macrophages into areas of allergen-exposed skin. Moreover, the preponderance of lymphocytes among the mononuclear cells in CHS (Figure 1) may be explicable by a broader spectrum of lymphocyte-attractant chemokines (IP-10, MIG, MCP-1, MDC, PARC, RANTES, TARC) than of monocyte-attractant chemokines (see Figures 7 and 10). Because, unlike for sensitization, epidermal Langerhans cells and dermal dendritic cells do not seem to be necessary for elicitation of CHS,² trafficking of this cell type was not investigated in the present study.

There is an obvious redundancy in expression of monocyte- and lymphocyte-specific chemokines during elicitation of CHS that has previously not been described. At first sight this seems to be a redundancy for robust output of the chemokine system as recently suggested by Mantovani.¹⁴ However, individual infiltrating lymphocytes and monocytes bear more than one receptor and many of the chemokines detected in CHS bind to different receptors,^{24,25} thus enabling a strong persistent stimulation of migrating effector cells despite a timely partial unresponsiveness of one or two receptor pathways after ligand binding. Therefore, one may speculate that the presence of a plethora of different chemokines with different receptor specificities may not only create robustness but also guarantees a rapid, nondelayed recruitment of cells to sites of evolving inflammatory challenge. Therefore, our data support the concept by Foxman and colleagues¹⁷ who have shown *in vitro* that sequential migration through two spatially distinct attractant fields can target leukocytes in a unique manner that is determined by both the nature and the sequence of attractants. The situation as exemplified in our study of inflamed skin is even more complex because leukocytes have to cross different compartments. Therefore, we propose a concept of multi chemokine-mediated recruitment of monocytes and T lymphocytes during elicitation of a CHS reaction as schematically illustrated in Figure 11.

Besides the chemokine expression profiles identifying the most active chemokine species in CHS our data may also explain the positioning of effector cells within the allergen-exposed skin. Importantly, IP-10 and MIG are thus far the only chemokines detected to be selectively chemoattractant for lymphocytes and expressed in su-

Figure 11. Multistep navigation of leukocyte trafficking during elicitation of CHS. The schematic drawing shows the time course and the spatial distribution of chemokine expression and leukocyte recruitment. During the early phase of CHS (<12 hours after allergen exposure) only one chemokine, MCP-1, is induced and expressed exclusively by single basal keratinocytes. In the intermediate phase (12 to 48 hours after allergen exposure) increasing strong dermal and particularly epidermal expression of MCP-1 and RANTES as well as dermal expression of MDC, PARC, and TARC is paralleled by pronounced recruitment of monocytes and T lymphocytes to the dermal compartment. During the late phase (48 to 96 hours after allergen exposure) expression of high levels of IP-10 and MIG in basal and suprabasal epidermal layers is associated with recruitment of lymphocytes to the epidermis. Monocytes reside in the dermis, which may be because of a lack of expression of monocyte-attractant chemokines in suprabasal layers of the epidermis. **Ar-**
rowheads point to the maximum of each chemokine gradient.

prabasal keratinocytes. Such an expression profile may explain why lymphocytes are initially detected in the dermis where they are potentially attracted by dermal RANTES, MCP-1, TARC, PARC, and/or MDC. At later time points (>48 hours) increasing MIG and IP-10 expression in suprabasal epidermal layers provides a strong chemoattractant signal for CXCR3-bearing lymphocytes that are subsequently recruited to suprabasal areas of the epidermis. In contrast, monocytes/macrophages attracted by MCP-1 and RANTES more or less reside in the dermis or immediately below the dermal-epidermal junction with some cell protrusions into the basal layer because suprabasal expression of monocyte-attractant chemokines is missing.

The relatively few neutrophils detected in CHS are possibly recruited on moderate expression of IL-8 and GRO α . Recent data obtained in the murine system suggested that Gro α expression and subsequent neutrophil infiltration are prerequisites for T cell recruitment and elicitation of the CHS reaction.²⁶ In the human CHS reaction, we could not only detect exclusive dermal Gro α expression but also simultaneous epidermal IL-8 and Gro α expression. This may explain why in humans neutrophils also infiltrate the epidermal compartment after 48 hours. Whether neutrophils recruited to the skin are a prerequisite for subsequent T cell trafficking as proposed by Dilulio and colleagues²⁶ remains to be elucidated for the human system.

Our data further provide evidence that in the initial phase of CHS basal keratinocytes as resident cells at the

interface between dermis and epidermis are the main contributors to chemokine expression. At this stage, inflammatory cells have not been recruited to the skin that argues, in agreement to earlier observations,⁴⁻⁶ that keratinocytes are directly stimulated rather than activated by cytokine-releasing infiltrating cells. The continuous expression of MCP-1 in the basal layer after 12 hours also argues against melanocytes, dendritic cells, or intraepidermal lymphocytes as a major source of epidermal MCP-1 message. Moreover, antigen-specific cells by chance patrolling through allergen-exposed skin areas are supposed to be rare;^{2,27} therefore, a vigorous activation of keratinocytes by those cells is very unlikely. The strong basal MCP-1 expression detected after 6 hours rather indicates that nonimmunological stimuli, most likely the intrinsic proinflammatory properties of the allergen itself, are responsible for such an early expression by keratinocytes. Analysis of skin organ cultures obtained from nonsensitized individuals revealed that epicutaneously applied contact haptens such as nickel chloride induce chemokine and adhesion molecule expression in the absence of specific T-cell-mediated immunity (our own unpublished observation). Our data clearly support the concept that allergens provide two distinct signals: beyond an antigen-specific signal a defined nonspecific, proinflammatory signal is delivered that is required to elicit a contact hypersensitivity reaction.² The hapten nickel chloride directly activates the transcription factor nuclear factor- κ B as well as p38 stress kinase-dependent signaling pathways that finally results in expression of adhesion molecules and chemokines.²⁸⁻³⁰ It is conceivable that different types of contact allergens may activate different cellular responses resulting in distinct chemokine expression patterns. For example, urushiol, the active agent in poison ivy, induces considerable IL-8 expression in keratinocytes.^{5,23} Further studies will have to focus on the heterogeneity of irritant properties of different contact haptens and its impact on the elicitation phase of CHS.

Because CHS is regarded as a T_{H1} response with T_{H1} -related IFN- γ -inducible chemokines MIG and IP-10 appearing late after allergen contact (>48 hours), the nonspecific inflammatory reaction induced by the irritative properties of allergens possibly precedes the allergen-specific immunological response by several hours and may furthermore accompany and amplify antigen-specific CHS. Recently, Flier and colleagues³¹ demonstrated that at later time points (>48 hours) MIG, IP-10, and IP-9 are essentially only detected in allergic but not in irritant patch-test reactions thus supporting our concept that at early time points the clinically invisible irritative inflammatory reaction is dominating whereas at later time points the immunological response evolves. During the later phase, strong expression of the IFN- γ -inducible chemokines IP-10 and MIG that is accompanied by a moderate expression of MIP-1 α/β may activate and preferentially attract T_{H1} cells that express CXCR3 and CCR5, respectively.³² In contrast, other T-cell-attracting chemokines detected show less preference for T_{H1} subtypes or are rather specific for T_{H2} subtypes such as TARC and MDC.³³ Thus, the chemokine profile may in-

fluence the pattern of T cell subsets recruited during inflammation and supports the concept by D'Ambrosio and colleagues³⁴ who hypothesize that different thresholds for T_{H1} and T_{H2} cells exist that differently localize T_H cell subsets in inflammation. Expression of more promiscuous T cell attractants such as MCP-1 and RANTES may explain the presence of bystander cells that are detected in CHS.²⁷

Taken together, our data provide evidence that 1) mononuclear cells in CHS encounter multiple chemoattractant signals in complex spatial and temporal patterns, 2) chemokines both mediate and integrate the irritant and allergen-specific pathways of CHS, and 3) a compartmentalized tissue such as the skin represents an ideal model to investigate the chemokine-mediated combinatorial control of leukocyte targeting in human tissue. Furthermore, our data provide evidence that inflammatory diseases such as CHS are characterized by their chemokine profile that may be a target for a specific anti-inflammatory intervention.

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