Expression of cytokines and chemokine receptors in the cutaneous lesions of erythema multiforme and Stevens–Johnson syndrome/toxic epidermal necrolysis

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Conflicts of interest
None declared.

Summary

Background Erythema multiforme (EM) and Stevens–Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) are caused by a dysregulation of cellular immunity. Objectives To evaluate further the potential role of certain cytokines and chemokine receptors in cutaneous lesions of patients affected by EM and SJS/TEN and to establish whether such diseases are polarized preferentially towards a T-helper (Th) 1 or Th2 pattern.

Methods Biopsy specimens from eight patients with EM, six with SJS/TEN and three healthy controls were stained for immunohistochemical examination using the alkaline phosphatase–antialkaline phosphatase method. The monoclonal antibodies used included those to tumour necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-2, IL-5, IL-13, receptor 3 for C-C chemokines (CCR3), receptor 3 for C-X-C chemokines (CXCR3) and CXCR4.

Results The SJS/TEN specimens showed significantly higher expression of all the cytokines and chemokine receptors (except CXCR3) tested than the EM specimens. Both lesional series showed significantly higher expression of all the receptors tested than the healthy control specimens, with the sole exception of a lower expression of CCR3 in EM specimens. Comparison between molecules associated with a Th1 or Th2 response revealed a predominance of Th1 response in EM and no significant imbalance between Th1 and Th2 in SJS/TEN.

Conclusions We have provided further evidence that TNF-α is strongly expressed in SJS/TEN lesions and therefore it may be involved in the epidermal necrosis featured in such diseases. IFN-γ may play an important role both in EM and SJS/TEN. IL-2, IL-5 and IL-13 may contribute to the cutaneous immunoinflammation in these diseases. Chemokine receptors may be involved strongly in the recruitment of inflammatory cells in lesional skin. In our cases we found a sharp polarization towards a Th1 pattern in EM, while the SJS/TEN lesions showed a mixed Th1/Th2 pattern.

Erythema multiforme (EM) and the Stevens–Johnson syndrome/toxic epidermal necrolysis (SJS/TEN) spectrum are characterized by polymorphous lesions affecting skin and/or mucosae with variable amounts of necrosis of basal keratinocytes and/or mucosal epithelial cells. EM and SJS/TEN are currently classified as distinct entities according to epidemiological, clinical, histopathological and aetiological differences.1–3 Although the pathogenesis of both conditions remains poorly understood, these dermatoses are presumably determined by a dysregulation of cell-mediated immunity.
resulting from either genetic predisposition or exogenous triggering factors, mainly herpes simplex virus (HSV) in EM and drugs in SJS/TEN.

The main pathogenetic mechanism in EM is presumably the activity of HSV- or drug-specific T-helper (Th) 1 lymphocytes, primarily via secretion of proinflammatory mediators and secondarily via expression of Fas ligand (FasL) and CD40 ligand (CD40L). On the other hand, SJS/TEN lesions are mainly determined by perforin-, granzyme B- and tumour necrosis factor (TNF)-α-secreting CD8+ lymphocytes, together with the activity of FasL+, CD40L+ T CD4+ lymphocytes. Macrophages and neutrophil granulocytes may also play an important role in enhancement of the cutaneous damage in both EM and SJS/TEN.

In order to better understand the pathogenetic mechanisms leading to the mucocutaneous lesions, some studies have attempted to evaluate the production of cytokines and chemokines in EM and SJS/TEN. Tissue immunohistochemical examinations showed an increased expression of TNF-α, interferon (IFN)-γ, interleukin (IL)-6, IL-10, and IL-18 in SJS/TEN lesions. Increased levels of TNF-α, IFN-γ, IL-10, IL-2, IL-6 (and its soluble receptor), IL-8 (and its soluble receptor) and IL-10 were found in the peripheral blood mononuclear cells (PBMC) from SJS/TEN patients. Significantly increased levels of MIG (macrophage IFN-γ-inducible gene), IFN-γ inducible protein (IP)-10, monocyte chemotactic protein (MCP)-1 and RANTES (regulated on activation normal T expressed and secreted) were found in EM specimens, while receptor 10 for C-C chemokines (CCR10) was expressed on PBMC from SJS/TEN patients.

The aim of our study was to evaluate the tissular levels and distribution of certain cytokines and chemokine receptors that had never been studied within cutaneous lesions of patients affected by EM and SJS/TEN, i.e. IL-2, IL-5, IL-13, CCR3, receptor 3 for C-X-C chemokines (CXCR3) and CXCR4. Moreover, we analysed the potential role of TNF-α and IFN-γ in these diseases. Finally, we attempted to establish clearly whether EM and SJS/TEN lesions featured a Th1 or Th2 pattern.

Methods

Patients and skin samples

The case series comprised eight patients with EM (four males, four females, aged 19–63 years, mean age 43 years) and six with SJS/TEN spectrum (one male, five females, aged 30–93 years, mean age 57 years). Diagnosis was made in all cases on the basis of clinical, histological, immunopathological and aetiological criteria. In particular, we diagnosed seven cases of EM minor, one of EM major, one of SJS, one of SJS/TEN overlap and four of TEN. Four EM cases were herpes-associated, two drug-associated (caused by oral contraceptives in one case, acetylsalicylic acid or macrolides in the other), but in two no triggering agents were discovered. All subjects with SJS/TEN were taking medication at the time of the eruption, i.e. vancomycin, meropenem (or ceftriaxone), trimethoprim–sulphamethoxazole (or norfloxacin), cloroquine, cyclophosphamide and paclitaxel (Taxol®). Following informed consent, we took lesional skin biopsies from the dorsum of the hands, palms or forearms of the EM patients and from the glutei or legs of the SJS/TEN patients. Again with informed consent, the surgical team of our hospital obtained for us control specimens of normal-appearing skin of three males (ages 35–50 years, mean age 45 years). All the skin specimens were immediately frozen at −80°C in liquid nitrogen.

Immunohistochemistry

Tissue specimens were cut into 5-μm-thick sections, which were stained immunohistochemically. The monoclonal antibodies included those to TNF-α (1 : 30), IFN-γ (1 : 100), IL-2 (1 : 100), IL-5 (1 : 50), IL-13 (1 : 30), CCR3 (1 : 50), CXCR3 (1 : 50) and CXCR4 (1 : 50), all supplied by R & D Systems (Minneapolis, MN, U.S.A.).

Before staining, frozen sections were air-dried and fixed in aceton (5 min). Immunolabelling was performed using rabbit antirat mouse bridging antibodies conjugated with alkaline phosphatase (1 : 10 dilution, 30 min; Dako, Copenhagen, Denmark) followed by incubation with murine alkaline phosphatase–antialkaline phosphatase complexes (1 : 30 dilution, 30 min; Dako). Negative control sections were incubated with nonimmune mouse sera.

Two independent ‘blind’ observers evaluated serial sections. For quantitative analysis, the stained cells were counted in three consecutive microscopic fields (250×) and then the average was calculated. Statistical analysis was performed with a Wilcoxon rank sum test. P < 0.05 was considered statistically significant.

Furthermore, the staining was quantitated using the following nomenclature to indicate the cell number per field: weak, between 0 and 10; moderate, between 10 and 20; and strong (or intense), greater than 20.

Results

Cytokines

TNF-α was weakly and focally expressed in EM perivascular dermis, being significantly less present than in SJS/TEN specimens (see Fig. 1), where it was distributed also in the subjunctional dermis, but more than in healthy ones.

IFN-γ+ cells were distributed focally in the superficial perivascular/interstitial dermis of EM specimens (Fig. 2). In SJS/TEN such molecules were strongly expressed in both perivascular and junctional dermis, some positive cells also showing an epidermal exocytosis. Statistical analysis revealed that IFN-γ+ cells showed significantly higher expression in SJS/TEN than in EM.
Another Th1-related cytokine, i.e. IL-2, was distributed in the perivascular dermis of all lesional specimens (Fig. 2); a moderate quantity in EM featured a less significant expression compared with the strong amount in SJS/TEN.

In the EM specimens the Th2-related cytokines IL-5 and IL-13 were very weakly expressed around dermal vessels and in the epidermis, practically negative in two of the eight cases studied (Fig. 3). However, in the SJS/TEN specimens, IL-5 and IL-13 stainings were strong and moderate, respectively, and significantly more evident than in EM, with a perivascular distribution in all cases.

Stainings for IFN-γ, IL-2, IL-5 and IL-13 were completely negative in the healthy controls specimens.

Finally, we tried to analyse the numerical data by summing Th1- and Th2-cytokine-positive cell counts and comparing them statistically. The Th1-related cytokines (IFN-γ and IL-2) were significantly overexpressed compared with the Th2 ones (IL-5 and IL-13) in EM (P = 0.0052), whereas the SJS/TEN lesions showed a mixed Th1/Th2 pattern (Wilcoxon rank sum test: not significant).

### Chemokine receptors

CCR3+ cells were found around the microvessels of lesional dermis in all cases. The expression of this surface receptor was very weak in EM but very strong—and significantly higher—in SJS/TEN (Figs 4, 5). There were no significant differences in distribution or numbers of CCR3 cells in EM and healthy control skin specimens.

CXCR3 was strongly distributed in the superficial perivascular dermis and in the basal/suprabasal epidermis in all lesional specimens. We found no significant differences between the disease groups, but in both the EM and SJS/TEN specimens the CXCR3+ cells significantly outnumbered those evidenced in healthy skin.

Comparison between the numbers of CXCR3+ cells (mainly Th1 lymphocytes) and CCR3+ cells (mainly Th2 lymphocytes) evidenced the Th1 pattern of EM lesions, while SJS/TEN lesions showed a mixed Th polarization (Wilcoxon rank sum test: not significant).

CXCR4+ cells were very weakly expressed in EM perivascular dermis, and two specimens were completely negative.
However, this receptor was strongly expressed in the perivascular and papillary dermis of all SJS/TEN specimens, except for those of one case that was negative. The SJS/TEN specimens presented significantly greater numbers of CXCR4+ infiltrating cells than the EM specimens.

Discussion

The recent recognition that EM and SJS/TEN are clinically and aetiologically distinct entities parallels accumulating experimental data that seem to indicate involvement of different pathogenic mechanisms in these two diseases. EM and SJS/TEN are presumably determined by a dysregulation of T-cell mediated immune mechanisms, in which soluble mediators such as cytokines⁶,¹⁵ and chemokines¹³,¹⁸ probably play a crucial role.

In our EM specimens, TNF-α, previously demonstrated only in drug-induced cases,⁶ was weakly produced in herpes- and drug-induced cases without significant differences. This finding allows one to consider TNF-α a poorly relevant mediator in EM lesions. On the other hand, our study confirmed that TNF-α represents one of the most important mechanisms of epidermal necrosis in SJS/TEN,¹¹,¹³ because it was strongly expressed in lesional perivascular dermis and the epidermis.

As shown previously by Kokuba et al.⁷ in herpes-associated cases of EM, we found that IFN-γ+ cells were distributed in the perivascular superficial dermis, almost colocalizing with CD4+ T lymphocytes. IFN-γ may be involved in activating...
macrophages and keratinocytes to release inflammatory molecules, upregulating major histocompatibility complex (MHC)-I and -II, inducing CD4 T-cell accumulation in the skin and favouring the differentiation of Th1 cells while inhibiting generation of Th2 cells. Moreover, we demonstrated intense expression of IFN-γ in the superficial dermis and epidermis of SJS/TEN lesions. These findings agree with those of Nassif et al., who found high levels of this molecule in TEN blister fluid. This observation leads to the hypothesis that IFN-γ, produced by either T CD4+ or T CD8+ lymphocytes, may play an important role in epidermal apoptosis, mainly by inducing overexpression of MHC-I on keratinocytes which makes them more susceptible to the action of drug-specific T CD8+ cytotoxic lymphocytes.
We found no data concerning the production of IL-2 in EM in the literature. This Th1-related cytokine has been demonstrated in the peripheral blood of SJS/TEN patients, but it is practically absent in the blister fluid.\textsuperscript{13} We evidenced IL-2 expression in the perivascular dermis of both EM (moderately) and SJS/TEN (strongly). IL-2 may exercise several proinflammatory functions via its receptor CD25,\textsuperscript{21} including lymphocyte proliferation and activation, in lesional skin in both diseases.

Th2-related cytokines, i.e. IL-5 and IL-13, were very weakly produced in EM, implying that they probably do not play a significant role in the inflammatory process. In contrast, IL-5 and IL-13 were intensely and moderately expressed in SJS/TEN lesions, respectively. The presence of such Th2 response may be related to drug sensitization, which might favour a pronounced Th2 reaction, similar to what happens in other cutaneous drug reactions.\textsuperscript{21} Thus, IL-5 and IL-13 may enhance cutaneous immunoinflammation in SJS/TEN, e.g. via recruitment and activation of eosinophils\textsuperscript{23} and IL-13 may enhance cutaneous immunoinflammation in SJS/TEN lesions may be related to downregulation aimed at self-limitation of the inflammatory process.

Chemokine receptors were expressed mainly by cells infiltrating the lesional perivascular dermis; immunostainings were weak in EM (except for CXCR3) and strong in SJS/TEN. CCR3 is preferentially expressed on Th2 lymphocytes, eosinophil granulocytes and neutrophils when stimulated by IL-2, IL-4,\textsuperscript{24} IL-5\textsuperscript{25} and IFN-\gamma.\textsuperscript{26}

Although RANTES, a CCR3 ligand, had been found to be elevated in EM lesions,\textsuperscript{18} the low number of CCR3+ cells we found in our EM series prevents any hypothesis favouring a primary role for this chemoattractant pathway.

Instead, the recruitment of the high amounts of Th2 lymphocytes and neutrophils\textsuperscript{7} in SJS/TEN lesions may be related somehow to the CCR3-mediated pathway, as we found intense immunostaining for this receptor. CXCR3, a cell-surface receptor mainly involved in chemoattraction and activation of Th1 cells,\textsuperscript{27} may play an important role in both diseases. In fact, many CXCR3+ Th1 lymphocytes may follow the MIG and IP-10 gradients present in EM lesions.\textsuperscript{18} CXCR3 may have the additional function of activating CD8+ T lymphocytes,\textsuperscript{28,29} CXCR4, expressed by T lymphocytes, monocytes and granulocytes,\textsuperscript{30} may contribute to the recruitment of the inflammatory-cell infiltrate, particularly in SJS/TEN lesions, where such molecules were well represented on infiltrating cell populations.

Finally, our analysis of intralesional expression of cytokines and chemokine receptors clearly evidenced a strong polarization towards a Th1 pattern in EM, as already documented by other authors.\textsuperscript{15,16} Instead, SJS/TEN lesions featured a mixed Th1/Th2 pattern, a finding that supplements that by Nassif et al.\textsuperscript{13} In fact, the latter hypothesized a Th1 polarization in TEN lesions by detecting high levels of IFN-\gamma and IL-18 in the blister fluid, but did not provide any data concerning in situ production of Th2-specific molecules.

In sum, on the basis of previous studies and the original data presented above, we conclude that HSV/drug-specific and Th1 lymphocytes are recruited in EM lesions (mainly via CXCR3-mediated chemoattraction) and then play a pivotal pathogenic role by secreting IFN-\gamma and IL-2. Instead, the key effectors in SJS/TEN are probably drug-specific cytotoxic T lymphocytes.\textsuperscript{10}

Furthermore, Th1 cells, recruited mainly via CXCR3 and secreting IFN-\gamma\textsuperscript{13} and IL-2, also play an important role in the tissular immunoinflammation. The role of Th2 cells (recruited via CCR3 and secreting cytokines, such as IL-5 and IL-13) in enhancing or regulating the inflammatory process awaits further clarification. The CD40L- and FasL-mediated pathways may represent other primary mechanisms by which activated T lymphocytes trigger SJS/TEN lesions.\textsuperscript{7}

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