Dynamics of the Cellular and Humoral Components of the Inflammatory Response Elicited in Skin Blisters in Humans

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Abstract

Skin blisters induced by suction on the forearm of normal volunteers provide a convenient model to study the inflammatory response in vivo in man. In our study, after removal of the roof of the blister, i.e., the epidermis, the exposed floor of the blister (dermal–epidermal interface) was bathed with 70% autologous serum using a multiwell skin chamber. Migration of leukocytes (90–95% neutrophils) into the chamber fluid was detectable within 3 h, and appeared to plateau at 16–24 h. Sampling of the dermal–epidermal interface revealed primarily mononuclear cells during the first 8 h of the inflammatory response; however, their prevalence at 24 h was greatly diminished due to neutrophil infiltration. Accompanying the cellular immune response was the accumulation of inflammatory mediators in the bathing medium. The accumulation of IFN-γ reached a plateau within 3 h; significant accumulations of the complement fragment, C5a, and of leukotriene B4 were also detected at 3-h. The accumulation of C5a did not peak until 5 h, whereas leukotriene B4 continued to accumulate through 24 h. IL-6 and IL-8 concentrations were minimal at 3–8 h but dramatic by 24 h while IL-1β, tumor necrosis factor-α, and granulocyte-macrophage colony-stimulating factor were undetectable within 3–8 h, but markedly elevated by 24 h. There was little accumulation of IL-4 and no accumulation of IL-1α or IL-2 during the 24-h period. The sequential appearance of mediators at an inflammatory focus suggests that a carefully regulated dynamic system is responsible for controlling the evolution of the inflammatory response. (J. Clin. Invest. 1992. 89:1734–1740.) Key words: cytokines • neutrophils • epidermis • interferon γ • C5a

Introduction

Development of inflammatory foci is accompanied by an extensive migration of neutrophils and mononuclear cells into the affected area. The cellular events have been characterized in man using skin window and blister chamber devices (1–3). However, the kinetics of the accumulation of inflammatory mediators at these sites have not been well described. Although neutrophil-specific (secondary) granule contents appear early in blisters fluid, there is a delayed appearance of neutrophil azurophil (primary) granule contents (4). Little has been published on the kinetics of the appearance of other soluble compo-
The blister floor was sampled by scraping with the edge of a sterile microslide over the floor of the blister, and then a smear for differential cell analysis was prepared. No attempts were made to quantitate the total number of cells present on the floor of the blister by tissue biopsy. Cells were identified by differential staining using a modified Wright stain (Diff-Quik®; Baxter Scientific Products, McGaw Park, IL), by nonspecific esterase staining (5), and by myeloperoxidase staining (6). Generally, 200 cells were counted and the values reported as the percentage of the cells counted. The entire field was counted in cases in which the number of cells was < 200. This approach provided a quantitative indication of cell content on the blister floor.

The levels of cytokines and inflammatory mediators in the various biological fluids were determined using commercially available kits. The following cytokines were determined by ELISA as described by the manufacturers without modification. Lower detection limits are included in parentheses. IL-1β (20 pg/ml) (Cistron Biotechnology, Pine Brook, NJ), IL-4 (45 pg/ml), IFN-γ (100 pg/ml) (Genzyme Corp., Boston, MA), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (2.8 pg/ml), IL-1α (7 pg/ml), IL-2 (55 pg/ml), IL-6 (3.5 pg/ml), IL-8 (18.1 pg/ml), tumor necrosis factor- α (TNF-α) (7.5 pg/ml) (R&D Systems, Inc., Minneapolis, MN).

Levels of endotoxin were determined by a chromogenic limulus amebocyte lysate assay (lower detection limit, 0.05 endotoxin units (EU)/ml) (Whittaker Bioproducts, Walkersville, MD). Samples assayed for endotoxin were diluted 1:5 with endotoxin-free water and then heated for 10 min at 70°C to inactivate inhibitors of chromogen development that were present in the serum. Samples were then further diluted 1:10 before analysis. The sensitivity of the assay was increased as described by the manufacturers. The detection of spiked endotoxin standards was > 90%.

The levels of leukotriene B4 (LTB4) were determined by radioimmunoassay (lower detection limit, 31 pg/ml) (Amersham Corp., Arlington Heights, IL). Samples for LTB4 analysis were extracted on a solid phase matrix of C2-bonded silica (Analytichem International, Harbor City, CA). Samples were acidified with HCl to pH 3, adsorbed to the column, and differentially eluted with H2O, 10% ethanol, and n-hexane. LTB4 was eluted with methyl formate. The sample was then dried under a stream of N2. The dry residue was redissolved in the assay buffer provided with the kit and assayed according to the manufacturer’s instructions. The recovery of a sample spiked with known quantities of LTB4 standard was 90%.

The levels of C5a were also determined by radioimmunoassay (lower detection limit, 10 ng/ml) (Amersham). The radioimmunoassay used to quantitate C5a does not differentiate between C5a and the C5a proteolytic cleavage product, C5a-desArg. In this experimental model, the predominant form of this primary inflammatory mediator was likely C5a-desArg. With all kits, the standard curves were analyzed using a 4-parameter curve-fitting program (7) and unknowns were calculated based on those curves.

Results

Cellular response. Cells accumulated in the neat blister fluid during the 1–2 h required for the suction blister to develop. Although these cells were not definitively identified, as shown in Fig. 1 A and B, these cells were few in number and generally nonspecific esterase-negative mononuclear cells. Additional histochemical analysis revealed that these cells were peroxidase negative. Removal of the roof of the blister and replacement of the neat blister fluid with 70% autologous serum (diluted with HBSS(−)) elicited a 3-log accumulation of cells in the chamber fluid (Fig. 1 A) within 18–24 h. As shown in Fig. 1 B, the leukocytes accumulating in the bathing medium were predominantly neutrophils (> 90%) with minimal contribution by either monocytes or lymphocytes. Bathing the skin lesion with physiological buffer (HBSS) resulted in very little migration of inflammatory cells into the chamber fluid.

To estimate the inflammatory cells accumulating at the dermal–epidermal interface, the floor of the skin lesion was sampled by scraping the lesion with the edge of a sterile glass slide. A representative experiment depicting the pattern of cell accumulation on the floor of the lesion is shown in Fig. 1 C. At t = 0 h, no cells were found on the floor of the lesion. During the first 5 h, monocytes and lymphocytes predominated at the dermal–epidermal interface, with neutrophils accounting for only 20% of the cells. By 8 h, neutrophils represented 50% of the cells, replacing the monocytes as the predominant cells present. By 24 h, the cellular distribution on the floor of the lesion was > 95% neutrophils, similar to that observed in the chamber fluid. This apparent decrease in the percentage of monocytes on the floor of the lesion likely reflects a dilution of the mononuclear cells by the extravasating neutrophils rather than a decrease in the total mononuclear cells at the site. Estimation of the total number of cells at the dermal–epidermal interface was not possible.

Inflammatory mediators. Because of the accumulation of various immune/inflammatory cell types on both the floor of the blister as well as within the chamber fluid, various inflammatory mediators/cytokines were expected to be present during the development of the inflammatory response. After the 1–2-h period required for the development of the blister, the neat blister fluid was harvested and analyzed for various inflammatory mediators. The mean levels of the complement fragment, C5a, IFN-γ, IL-8, and IL-1β in neat blister fluid (Fig. 2, A–D) were increased 430, 250, and 640%, respectively, compared with those observed in plasma obtained simultaneously from the same subject. The elevation in IL-1β, although statistically significant, reflected merely a change from an undetectable level of IL-1β to the lowest detectable level by the immunoassay. In contrast, the level of IL-4 in the neat blister fluid was significantly lower than that observed in plasma (Fig. 2 E). Levels of IL-6, IL-1α, TNF-α, LTB4, IL-2, and GM-CSF in neat blister fluid were not different from plasma (Fig. 2, F–K).

After harvest of the neat blister fluid and removal of the roof of the blister, the exposed floor of the blister was bathed in 70% autologous serum using the multiwell chamber. Significant accumulations of IFN-γ, C5a, and LTB4 were detected at 3 h (Fig. 3 A). The accumulation of IFN-γ (700 pg/ml) peaked within 3 h, and was maintained throughout the course of the incubation (Fig. 3 A). The accumulation of C5a, however, did not peak (30 nM/ml, 2.5 nM) until 5 h, whereas LTB4 continued to accumulate, throughout the study, totaling 2.5 nM/ml (7.5 nM) at 24 h. Although IL-8 and IL-6 were detected during the early phase (3–5 h) of the inflammatory response (Fig. 3 B) a much more dramatic accumulation of these mediators occurred between 8 and 24 h. In contrast, although only low levels of IL-1α (see Fig. 2), and no TNF-α, and GM-CSF were detectable in the early phase of the inflammatory response, between 8 and 24 h a dramatic increase of these mediators was observed (Fig. 3 C). Despite the dramatic elevation in the level of IL-1α at 24 h, the level of IL-1α did not change during the course of the incubation (Fig. 3 D). A small but detectable increase in the level of IL-4 was noted at 24 h. The level of IL-2

1. Abbreviations used in this paper: EU, endotoxin units; GM-CSF, granulocyte-macrophage colony-stimulating factor; LTB4, leukotriene B4; TNF-α, tumor necrosis factor-α.
Figure 1. Accumulation of leukocytes at the inflammatory locus. (A) Migration of leukocytes into both the neat blister fluid and the chamber fluid (70% autologous serum) bathing the dermal–epidermal interface vs. time. The dotted line designates the interval after the neat blister fluid was harvested, the epidermis removed, and the dermal–epidermal interface bathed with the chamber fluid. Each point represents the mean±SEM (n = 3–23). NBF, neat blister fluid. (B) was undetectable throughout the course of the incubation. It should be noted that although trace contamination of endotoxin was present in the 70% serum bathing the skin lesion, there was no apparent accumulation of endotoxin at the inflammatory locus in the 24-h sample (2.0±0.4 EU/ml [range 1.5–3.3 EU/ml] equivalent to 0.1 ng/ml).

**Systemic levels of mediators.** Because of the elevated levels of the inflammatory mediators found in the experimental inflammatory foci, it was necessary to ascertain whether this accumulation of mediators reflected systemic increases, or was indicative of a localized phenomenon. Plasma was collected at t = 0 and at t = 24 h, a time when the inflammatory mediators were present locally at their highest observed levels. Despite the accumulation of inflammatory mediators in the medium bathing the skin lesion, there was no detectable accumulation of any of these inflammatory mediators in the systemic circulation (data not shown).

**Response to heat-inactivated serum.** Bathing the skin lesion with heat-treated 70% autologous serum (30 min at 56°C) resulted in a delayed migration of cells into the bathing medium (Fig. 4A). This delay was most pronounced at t = 5 h; by 16–24 h, the migration of cells into the bathing medium had reached the same level as the control. The delayed cell migration correlated with a significant delay in the accumulation of C5a in the medium bathing the skin lesion. The accumulation of two other chemoattractants, LTB4 and IL-8, was unaffected when the skin lesion was bathed with heat-treated 70% autologous serum. These findings suggested that C5a (or C5a desArg) might be the major chemoattractant in the initial phases of this model of the inflammatory response.

During the later phase of the inflammatory response (16–24 h), the accumulation of both IL-6 and TNF-α was inhibited by 80% when the skin lesion was bathed with 70% heat-treated serum rather than 70% serum (IL-6, 6.6 nM/ml in heat-treated serum vs. 35.2 nM/ml in control serum, P < 0.001; TNF-α, 27.0 pg/ml in heat-treated serum vs. 170 pg/ml in control serum, P < 0.05). Although the mechanism for these suppressed responses with heated serum remains an enigma, the data suggested that the accumulation of IL-6 and TNF-α in vivo required a heat-labile serum component.

**Discussion**

We have characterized in man the kinetics of the accumulation of various inflammatory mediators at an experimental inflammatory focus, a skin lesion induced by suction. The possible sources of many of these inflammatory mediators in resident skin tissue has been recently reviewed (8). Epidermal tissue consists primarily of keratinocytes interspersed with occasional dendritic cells, Langerhans cells, and melanocytes. The underlying dermis consists primarily of extracellular matrix, e.g., col-
lager, but its cellular contents include fibroblasts, macrophages, and T cells, as well as capillary endothelial cells associated with the microvasculature. Infiltrating inflammatory monocytes and neutrophils are also possible sources of the complex cytokine response observed in this experimental inflammatory focus. Our qualitative data from scrapings of the blister floor indicate different cells comprise the tissue and fluid compartments of the inflammatory focus, perhaps reflecting different expression of adhesion proteins by cells in the two compartments. Although endotoxin could not explain the presence of mediators in neat blister fluid, a contribution of trace endotoxin detected in samples of fluid bathing blister lesions to the subsequent cytokine response cannot be excluded.

IFN-γ appeared to be one of the primary mediators in this model of the inflammatory response. The increased level of IFN-γ in neat blister fluid compared with plasma suggested that accumulation of IFN-γ occurred simultaneously with the development of the blister. Furthermore, accumulation of IFN-γ in the bathing medium reached a maximum concentration at 3 h, before most other cytokines were even detected. The concentration of IFN-γ measured in neat blister fluid (up to 430 pg/ml) was in the same order of magnitude as that required to stimulate human macrophage superoxide anion production in vitro (9). Thus far, only T lymphocytes and natural killer cells have been identified as sources of IFN-γ. The cells found in neat blister fluid were nonspecific esterase-negative, peroxidase-negative mononuclear cells. These data suggested that T lymphocytes, possibly skin-associated T cells (10), were a likely source for the production of IFN-γ. Possible targets of IFN-γ action were keratinocytes, whose surface expression of

Figure 2. Comparison of inflammatory mediators/cytokines in plasma vs. neat blister fluid. A–K show the levels of inflammatory mediators that were detected in plasma vs. neat blister fluid. (A) C5a; (B) IFN-γ; (C) IL-8; (D) IL-1α; (E) IL-4; (F) IL-6; (G) IL-1α; (H) TNF-α; (I) LTB4; (J) IL-2; and (K) GM-CSF. The data were analyzed by paired Student’s t test and the level of significance is indicated in the panels where significant differences were found. (c) Whole plasma; (●) neat blister fluid.
the lymphoid trafficking molecule, intercellular adhesion molecule 1, has been shown to be upregulated by treatment with IFN-γ (11, 12).

The chemotaxant, C5a (or C5a-desArg), was elevated in the neat blister fluid compared with plasma concentration. A comparable elevation of C5a was observed in a similar study that used a skin chamber technique (13). The concentration of C5a attained in the neat blister fluid was similar to that required to cause accumulation and adherence of neutrophils in the microvasculature after topical application in the hamster cheek pouch (14). Since neat blister fluid contains no detectable endotoxin, the accumulation of C5a in fluid of the sterile blister was unrelated to complement activation by exogenous endotoxin. After unroofing of the blister, further C5a accumulation could have resulted from complement activation by the trace amounts of endotoxin contamination. Replacement of the 70% serum in the bathing fluid with 70% heat-treated serum caused a significant delay in the accumulation of C5a and neutrophils as well as IL-6 and TNF-α, suggesting an important role for C5a in the early phase of neutrophil accumulation in this experimental model of inflammation.

Although not elevated in neat blister fluid, LTB₄ accumulated in the fluid bathing the skin lesion. Only neutrophils and monocytes/macrophages are thought to generate the 5-lipoxygenase product, LTB₄. Although other cells have the same 5-lipoxygenase activity, they process the initial product, LTA₄, to its glutathione adduct, LTC₄, and its subsequent metabolites. The rate limiting step for this reaction is generally thought to be the availability of the substrate, arachidonic acid. Neutrophils exposed to C5a generated LTB₄, but only if arachidonic acid were provided exogenously (15). In the study described here, the accumulation of C5a was delayed in 70% heat-treated serum while the accumulation of LTB₄ was unaffected. This suggested that either the accumulation of these two inflammatory mediators occurred independently, or the threshold level of C5a required for LTB₄ generation was very low and already exceeded.

The level of IL-8 was significantly higher in neat blister fluid (up to 2.9 ng/ml) than in plasma and was comparable to that required to stimulate chemotaxis of both neutrophils and T cells in vitro (16). However, the level of IL-8 in neat blister fluid represented only 1% of the level observed in the bathing medium at 24 h. Marginal accumulations of IL-8 and IL-6 in the medium bathing the skin lesion were detected at 3 and 5 h, respectively. Dramatic accumulations of both cytokines occurred from 8 to 24 h. In a similar study using a skin chamber technique, a comparable accumulation of IL-8 was observed by 24 h (13). Both IL-8 and IL-6 are produced by monocyte/macrophages. IL-8 is also produced by fibroblasts, T cells, and endothelial cells (17), as well as phagocytosing neutrophils (18). IL-8 is also thought to be produced by keratinocytes (8). Large amounts of IL-8 have been isolated from psoriatic lesions (17).

**Figure 3. Accumulation of inflammatory mediators/cytokines at the inflammatory locus.** (A–D) Accumulation of the indicated inflammatory mediators in the medium bathing the exposed dermal–epidermal interface vs. time. In each panel, the value at t = 0 represents the endogenous background level found in 70% autologous serum. The break in the x axis represents the time at which the 70% autologous serum was added to the chamber. Each point represents the mean±SEM (n = 3–19). For clarity, the data in A and C have been offset slightly.
and intradermal injection of IL-8 into normal human volunteers caused perivascular neutrophil infiltration (19). IL-6 is also produced by T cells, fibroblasts (20), and neutrophils (21), and has been found to be highly expressed by keratinocytes in psoriatic skin (22). Furthermore, skin, particularly injured skin (thermal injury, ultraviolet irradiation), is a major site for the production of IL-6 (23).

IL-1β was significantly elevated in the neat blister fluid compared to plasma. The concentration of IL-1β observed, although low, represented an order of magnitude concentration higher than that required to stimulate biological effects in vitro (24). Accumulations of IL-1β (but not IL-1α), TNF-α, and GM-CSF were not detected until after 8 h. The antibody used in the enzyme-linked immunosorbent assay for IL-1β does not distinguish between the inactive, 31 kD, pro-form of the IL-1β and the active, 17 kD, native form. Only three cell types have so far been shown to contain IL-1β convertase activity: monocyte/macrophage, the neutrophil, and the mast cell (25). Neutrophils have been shown to produce IL-1α and IL-1β (26–28). Keratinocytes and fibroblasts have been reported to produce the 31-kD inactive form of IL-1β but lack IL-1β convertase activity to process it to the active form (25). The presence of monocyte/macrophages and neutrophils at the lesion could promote conversion of pro-IL-1β to the active moiety. IL-1α, thought to be more cell associated than IL-1β (24), did not accumulate into either the neat blister fluid or the medium bathing the skin lesion. TNF-α is produced primarily by monocyte/macrophages, but has recently been reported to be released by neutrophils exposed to endotoxin (29) or to Candida albicans (30). GM-CSF is produced by T cells, monocyte/macrophages, keratinocytes (8), fibroblasts (31), and even endothelial cells (32). In many instances, the production of these “secondary” cytokines required another initiating “primary” signal. Although it is interesting to speculate that IFN-γ and C5α represented the primary signals, thus far, there is no evidence to indicate that the production of these secondary cytokines was in fact governed by these primary mediators.

Other mediators have yet to be measured in this model. It has recently been shown that neutrophils have receptors for transforming growth factor-β that mediate neutrophil chemotaxis (33), and transforming growth factor-β may play a role in the recruitment of neutrophils to inflamed synovial tissue (34). Other potential inflammatory mediators involved in the regulation of the focal inflammatory response are the growth factors involved in wound healing, particularly platelet-derived growth factor and fibroblast growth factor.

Despite the significant accumulations of inflammatory mediators that were observed at the experimental inflammatory

Figure 4 Effect of heat-treated serum on the accumulation of leukocytes and inflammatory mediators at the inflammatory locus. Open bars, 70% serum; hatched bars, 70% heat-treated serum. (A) Accumulation of leukocytes into either 70% serum or 70% heat-treated (56° for 30 min) serum at t = 5 and t = 24 h. Each bar represents the mean±SEM (n = 11–14). The indicated level of significance was determined by Student’s t test (70% serum vs. 70% heat treated serum). (B–D) Accumulation of chemoattractants ([B] C5α; [C] LTB4; and [D] IL-8) into either 70% serum or 70% heat-treated serum at t = 5 and t = 24 h. The values at t = 0 represent the endogenous levels of the chemoattractants detected in either the normal or heat-treated serum. Each bar represents the mean±SEM (n = 7–19). The level of significance was determined as indicated in A.

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focus, these accumulations were not reflected in higher levels in the systemic circulation. This indicates that the changes in mediator concentrations observed in the experimental inflammatory focus used in these studies is a localized phenomenon.

Finally, the presence of a cytokine does not imply de facto that its activity is present. Negative regulators of cytokine activity, such as soluble receptors or receptor antagonists (such as the IL-1 receptor antagonist) have already been described and may be present. Neutrophils constitutively produce a specific IL-1 inhibitor (35). Undoubtedly, other specific and nonspecific inhibitors will be identified that add greater complexity to the regulation of inflammation. Similarly, the inability to detect a cytokine does not exclude the possibility that local effective concentrations may play an important role in mediating inflammation. The inflammatory response reflects the dynamic accumulation of a number of inflammatory mediators, and the observed effects in vivo reflect the net effect of all the positive and negative mediators being produced at the inflammatory focus.

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