

Role of mast cells in the onset of IgE-mediated late-phase cutaneous response in mice

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Background: In mice that are passively sensitized to IgE, cutaneous antigen challenge produces a biphasic response with peaks at 1 and 24 hours after challenge.

Objective: We investigated the role of mast cells in the IgE-mediated late-phase reaction in mice.

Methods: We histologically and ultrastructurally investigated the morphologic changes of mast cells during the biphasic responses.

Results: Degranulation of mast cells, which was observed between 4 and 24 hours after challenge, reached a peak at 8 hours. Piecemeal degranulation was seen during the immediate phase reaction. The number of IL-6-positive mast cells was increased after 4 hours in both IgE-sensitized and unsensitized mice, but positive cells showed a greater increase in sensitized mice and reached a peak after 8 hours. With in situ hybridization experiments, mast cells were positive for IL-6 messenger RNA at 6 hours after challenge.

Conclusion: These findings indicate that anaphylactic degranulation of mast cells and the expression of IL-6 mRNA within 4 hours after antigen challenge are important for the onset of the late-phase allergic cutaneous reaction in mice. (*J Allergy Clin Immunol* 2000;106:S91-8.)

Key words: Mast cells, degranulation

In patients with allergy, an immediate phase reaction (IPR) occurs within 60 minutes of allergen challenge and is followed by a late-phase reaction (LPR), which appears after 3 to 48 hours.¹ The LPR is characterized by an infiltration of inflammatory cells and an increase in vasopermeability of various tissues including skin, lungs, nose, and eyes. LPR is of interest because of its similarity to the clinical manifestation of chronic allergic diseases.^{2,3}

To study the mechanism of allergic inflammation, the role of inflammatory cells and mediators has been assessed in rodent LPR models. Several immunologic responses have been reported to cause a cutaneous LPR in mice.^{4,5} Because IgE-mediated reactions are the most common, we previously established an IgE-mediated biphasic cutaneous response model in mice⁶ and used it to investigate the LPR, especially the role of inflammatory cells and mediators.

Abbreviations used

AND: Anaphylactic degranulation
DNFB: 2,4-Dinitrofluorobenzene
FcεRI: High-affinity IgE receptor
IPR: Immediate phase reaction
LPR: Late phase reaction
PMD: Piecemeal degranulation
SSC: Saline sodium citrate

Mast cells appear to be involved in both the IPR and the LPR in IgE-mediated allergic reactions. Some reports, including our own, have suggested the participation of mast cells in the cutaneous LPR in mice.⁷ Katayama et al⁸ reported that mast cell-deficient WBB6F1-W/W^v and WCB6F1-SI/SI^d mice, which had been passively sensitized with monoclonal IgE, showed no LPR to cutaneous antigen challenge.

Mast cells undergo degranulation after high-affinity IgE receptor (FcεRI)-mediated activation or other stimuli, which results in the release of histamine, proteoglycans, neutral proteases, and lipid-derived mediators.^{9,10} Mast cells also produce a wide variety of cytokines after FcεRI-mediated activation, and such production of mediators or cytokines by mast cells may be important in the pathogenesis of inflammatory diseases. Cytokine production is not merely an acute response to IgE-mediated activation but persists for many hours after mast cell activation by a variety of stimuli,^{11,12} and the production of some cytokines by mast cells is not necessarily associated with the release of preformed mediators.

IL-6 is a multifunctional cytokine that regulates immune and inflammatory processes.¹³ It can be produced by various cells, including fibroblasts, macrophages, and T and B lymphocytes.¹⁴ In the mouse, IL-6 is produced in the skin as part of the passive cutaneous anaphylaxis and is also produced by several mast cell lines in response to immunologic stimuli.^{15,16} Therefore, IL-6 production may be an important indicator of mast cell activation.

The present study was conducted to investigate the role of mast cells in the IgE-mediated cutaneous LPR in mice. We investigated the morphologic changes of mast cells during IPR and LPR histologically and ultrastructurally. In situ hybridization and immunohistologic methods were also used to examine the activation of mast cells and to measure the production of IL-6 messenger RNA and protein.

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METHODS

Mice

Female specific-pathogen-free BALB/c Cr Slic mice were obtained from Japan SLC (Shizuoka, Japan) and used at 8 to 10 weeks of age. They were housed in plastic cages in an air-conditioned room at 24°C, fed a standard laboratory diet, and given water as desired. All experiments were performed according to a guideline for the care and use of experimental animals made by the Japanese Association for Laboratory Animal Science in 1987.

Antibodies and other reagents

Monoclonal anti-dinitrophenol IgE was prepared from EC-1 cells.⁶ A rat anti-mouse IL-6 mAb was purchased from Genzyme (Cambridge, Mass), and biotinylated polyclonal anti-rat rabbit IgG was obtained from Sigma Chemical Co (St Louis, Mo). 2,4-Dinitrofluorobenzene (DNFB) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and was dissolved in acetone-olive oil (3:1).

IgE-mediated passive cutaneous reaction

The mice received an intravenous injection of 1.0 mL of anti-dinitrophenol IgE. Twenty-four hours after sensitization, a skin reaction was elicited with the application of 25 μ L of 0.15% DNFB solution to both ears; the acetone-olive oil vehicle was applied to control mice. The ear thickness was measured with an engineer's micrometer (Upright Dial Gauge; Peacock, Tokyo, Japan), and the net increase in thickness after antigen challenge was calculated by subtracting the value measured immediately before challenge. Our preliminary study showed that the vehicle did not affect the ear thickness.

Histologic features

Mice were killed by cervical dislocation before or after DNFB challenge. Ears were removed and immediately immersed in 4% paraformaldehyde-0.1 mol/L phosphate buffer (pH 7.4). The specimens were trimmed and fixed in this solution overnight at 4°C. Tissues were dehydrated in a graded ethanol series and embedded in paraffin. Then 4 μ m sections were cut and stained with hematoxylin and eosin or May-Grunwald Giemsa stain for histologic examination.

Electron microscopy

Some ear specimens were cut into small blocks and fixed for 4 hours at 4°C in a mixture containing 2% paraformaldehyde, 2.5% glutaraldehyde, and 25 mg CaCl_2 in 0.1 mol/L sodium cacodylate buffer (pH 7.4). The tissues were postfixed for 2 hours in 2% collidine-buffered osmium tetroxide, stained en block with uranyl acetate for 2 hours at room temperature, dehydrated in a graded ethanol series, and embedded in a resin, Epok 812 (Oken Shoji, Tokyo, Japan). Thin sections were prepared, stained with lead citrate, and examined under an electron microscope (JEM1200EX).

Immunohistochemistry

IL-6 was detected by the avidin-biotin-complex peroxidase method. Briefly, paraffin sections (4- μ m thick) were pretreated with 3% H_2O_2 in methanol for 5 minutes and subsequently incubated with 1% skim milk in 0.01 mol/L PBS (pH 7.4) for 20 minutes. Sections were then treated with rat anti-mouse IL-6 mAb (1:100) for 2 hours at room temperature, followed by biotinylated antibody (1:300) for 30 minutes. Next, sections were treated with avidin-biotin-complex (Vectastain ABC kit; Vector Laboratories Inc, Burlingame, Calif) for 30 minutes. Peroxidase activity was detected with 0.05%, 3,3'-diaminobenzidine tetrahydrochloride in 0.05 mol/L Tris-HCl (pH 7.6) containing 0.01% H_2O_2 and 0.065% sodium azide. Finally, slides were stained with alcian blue (pH 2.5) and counterstained with neutral red to identify mast cells.

In situ hybridization

The oligonucleotide probes used were synthesized by Takara Shuzo Co (Shiga, Japan). The 2 IL-6 antisense probes were 45 bases long and complementary to bases 523 to 567 (MIL6A) and 8 to 52 (MIL6B) of mouse IL-6.¹⁷ The 5'-ends of these probes were labeled with digoxigenin.

Paraffin sections were deparaffinized and treated with 0.2 N HCl for 20 minutes. After being washed in PBS, sections were treated with 10 mg/mL proteinase K (Wako Pure Chemical, Osaka, Japan) for 10 minutes at 37°C and then immersed in 4% paraformaldehyde for 5 minutes. Then the sections were treated with 2 mg/mL glycine in PBS for 30 minutes. After being washed in double-distilled water, the sections were treated with 50% deionized formamide in 2 $\times$ saline sodium citrate (SSC) for 1 hour and incubated overnight at 40°C with hybridization buffer containing the digoxigenin-labeled probe (200 pmol/L). After hybridization, sections were dipped twice in 50% formamide in 2 $\times$ SSC, rinsed in the same buffer (twice for 1 hour at 40°C and then for 30 minutes at 40°C), rinsed in 50% formamide in 1 $\times$ SSC (for 30 minutes at 40°C and then 30 minutes at room temperature), and rinsed in buffer A (0.1 mol/L Tris, 0.15 mol/L NaCl; pH 7.5). The labeled hybrids were processed with an alkaline phosphatase-labeled anti-digoxigenin antibody (Boehringer-Mannheim). The sections were then rinsed in buffer A (3 $\times$ 5 minutes) and in buffer B (0.1 mol/L Tris, 0.1 mol/L NaCl, 0.05 mol/L MgCl_2 , 5 minutes). Alkaline phosphatase activity was triggered with 0.33 mg/mL nitroblue tetrazolium and 0.16 mg/mL 5-bromo-4-chloro-3-indolyl phosphate in buffer B containing 0.1 mol/L levamisole as the chromogen. The reaction was stopped when the sections were rinsed for 5 minutes in PBS followed by 5 minutes in double-distilled water. Finally, sections were mounted with an aqueous mounting medium (Gel/Mount, Biomedica, Calif) without counterstain.

Quantitative analysis and statistics

The number of degranulated and IL-6-positive or IL-6-negative mast cells (alcian blue-positive cells) was counted under a light microscope (at least 3 ear sections per mouse), with more than 500 mast cells being examined in each preparation. The intensity of the IL-6 immunoreactivity of mast cells was classified into 3 grades, negative, weakly positive, and strongly positive. Only strongly positive cells were defined as IL-6-positive mast cells. Statistical differences of the degranulation or IL-6 immunoreactivity of mast cells were assessed with the χ^2 -test or 1-way ANOVA followed by a post-hoc Scheffe's test.

RESULTS

Ear thickness

The changes of ear thickness in passively sensitized mice showed 2 peaks after DNFB challenge (Fig 1, A). The first peak was at 1 hour, and the second was at 24 hours after the challenge, which represented the IPR and LPR, respectively.

Histologic findings

At 30 minutes to 1 hour after the DNFB challenge, slight dermal edema and vasodilatation were observed in the ears (Fig 2, A and B). At 4 and 8 hours, mild inflammatory cell infiltration (mainly neutrophils and a few macrophages) and focal edema were observed in the dermis (Fig 2, C). At 24 and 48 hours, cellular filtrates that consisted of neutrophils and a few eosinophils were seen in both the dermis and epidermis (Fig 2, E and F). Dermal edema, vasodilata-

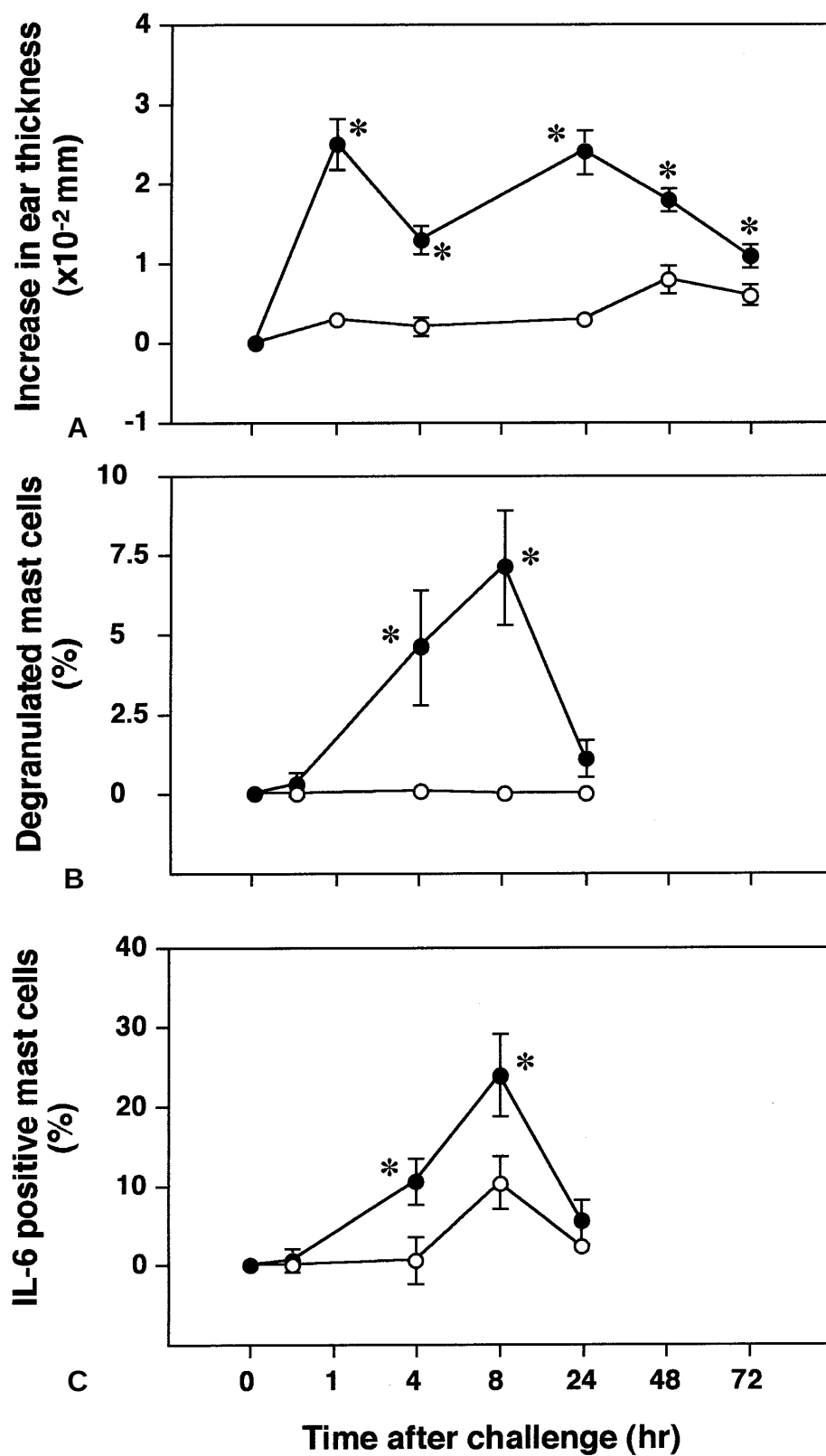


FIG 1 A, Time course of the IgE-mediated increase in ear thickness in BALB/c mice. Mice received an intravenous injection of 1.0 mL of anti-dinitrophenol IgE 24 hours before skin challenge with 0.15% DNFB. B, Degranulation of mast cells in edematous lesions 4 to 24 hours after DNFB challenge. C, The number of strongly IL-6-positive mast cells in edematous lesions 4 to 24 hours after DNFB challenge. **P* < .01.

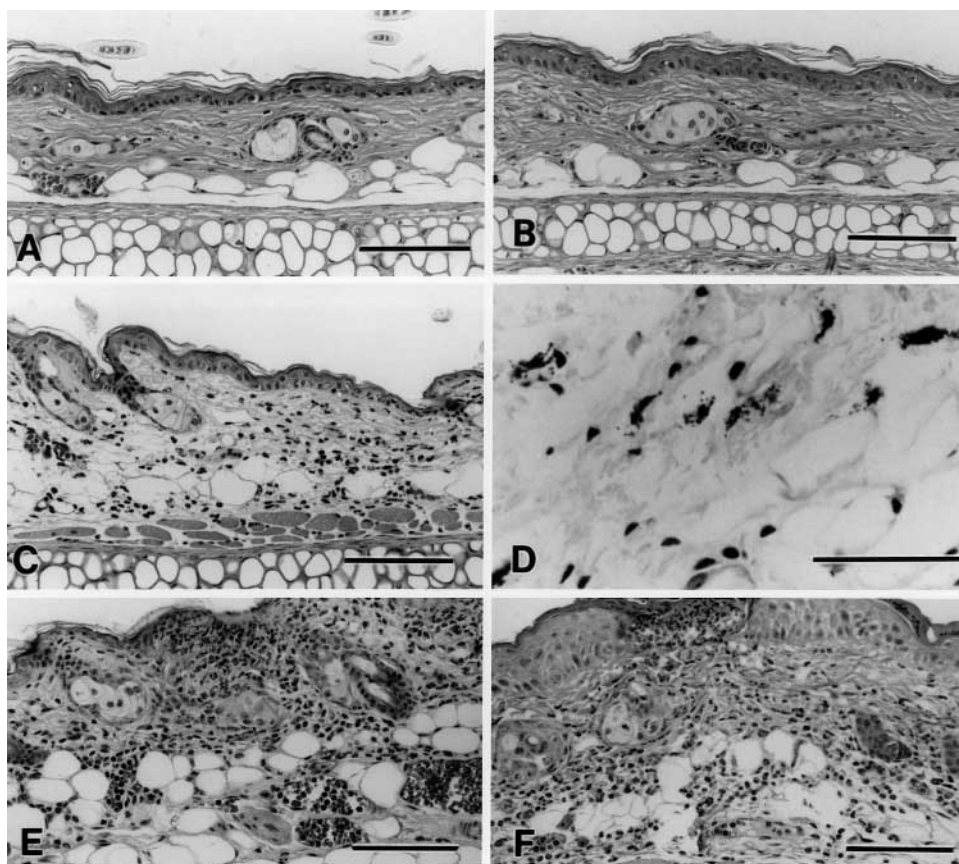


FIG 2. Histologic changes in the ears of BALB/c mice alter DNFB challenge. Mice were sensitized with a monoclonal anti-dinitrophenol IgE. After 24 hours, 0.15% DNFB was applied to both ears. **A**, No treatment. **B**, At 1 hour after DNFB challenge. **C** and **D**, At 8 hours after DNFB challenge. **E**, At 24 hours after DNFB challenge. **F**, At 48 hours after DNFB challenge. Bar = 100 μ m (**A-C**, **E**, and **F**) or 50 μ m (**D**). Hematoxylin and eosin stain (**A-C**, **E** and **F**), May-Grunwald Giemsa stain (**D**); original magnification, $\times 200$ (**A-C**, **E**, and **F**) or $\times 400$ (**D**).

tion, and epidermal hyperplasia were also present. At 72 hours, the epidermal hyperplasia showed a peak, but the other changes were reduced. Acetone-olive oil (vehicle control) caused no histologic changes.

May-Grunwald Giemsa-stained sections showed mast cell degranulation in the edematous areas at 4 hours and to a larger extent at 8 hours after DNFB challenge (Fig 1, *B*). Some of the degranulated mast cells were degenerated and were surrounded by neutrophils (Fig 2, *D*). At 24 hours after the challenge, however, there were few degranulated mast cells within the dermis.

Ultrastructural changes of mast cells

In comparison to normal mast cells (Fig 3, *A*) mast cells detected at 15 minutes to 1 hour after the DNFB challenge featured enlarged secretory granules filled with altered contents, which were partially empty or showed diminished electron density (Fig 3, *B*). There was no evidence of the extrusion of granular contents or the fusion of granule membranes. These changes were typical of piecemeal degranulation (PMD). Mast cells detected at 4 and 8 hours after the challenge showed typical anaphylactic degranulation (AND). In these mast cells, multiple

swollen and altered membrane-free granules were extruded along with some components of the cytoplasmic organelles (Fig 3, *C*). The remaining granules were enlarged and were fused to form intracytoplasmic degranulation channels that were filled with altered and swollen granule contents. We also observed that neutrophils attached to mast cells that showed AND and that phagocytized extruded mast cell granules (Fig 3, *D*). At 24 hours after the challenge, the mast cells contained a few enlarged and fused granules, but there was no extrusion of granules.

Immunohistochemistry for IL-6

IgE-mediated induction of IL-6 production by dermal mast cells in the ears of sensitized mice was observed by immunoperoxidase staining. In untreated mice, only 5% of all mast cells (alcian blue-positive cells) showed IL-6 immunoreactivity. A significant increase in the number and staining intensity of IL-6 immunoreactive mast cells was seen at 4 hours after the DNFB challenge, with the staining peaking at 8 hours (Fig 1, *C*). Strongly positive mast cells located in or around the edematous site accounted for 11% and 24% of all mast cells at 4 and 8

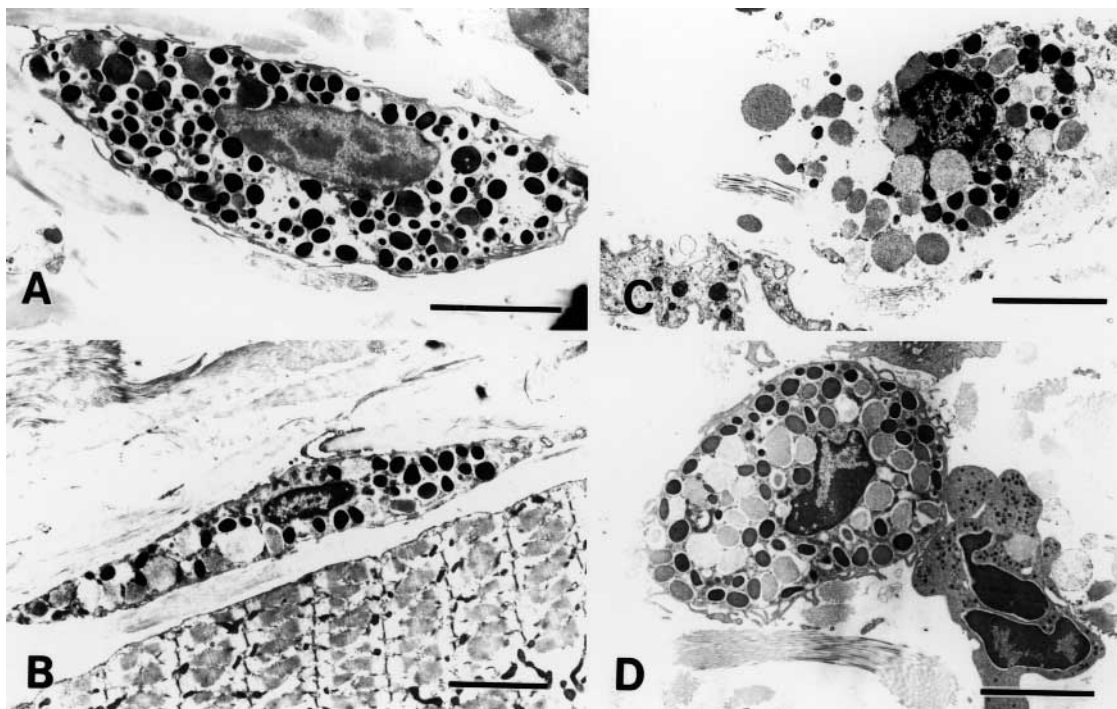


FIG 3. Ultrastructure of mast cells after DNFB challenge. **A**, Normal mast cell. **B**, PMD; some secretory granules are enlarged and show altered contents at 30 minutes after challenge. **C**, AND; multiple swollen and altered granules have been extruded at 8 hours after challenge. **D**, A neutrophil is attached to a mast cell showing AND and phagocytosis of extruded granules by neutrophil at 8 hours after challenge. Bar = 5 μ m. (Lead citrate stain; original magnification, $\times 5,100$.)

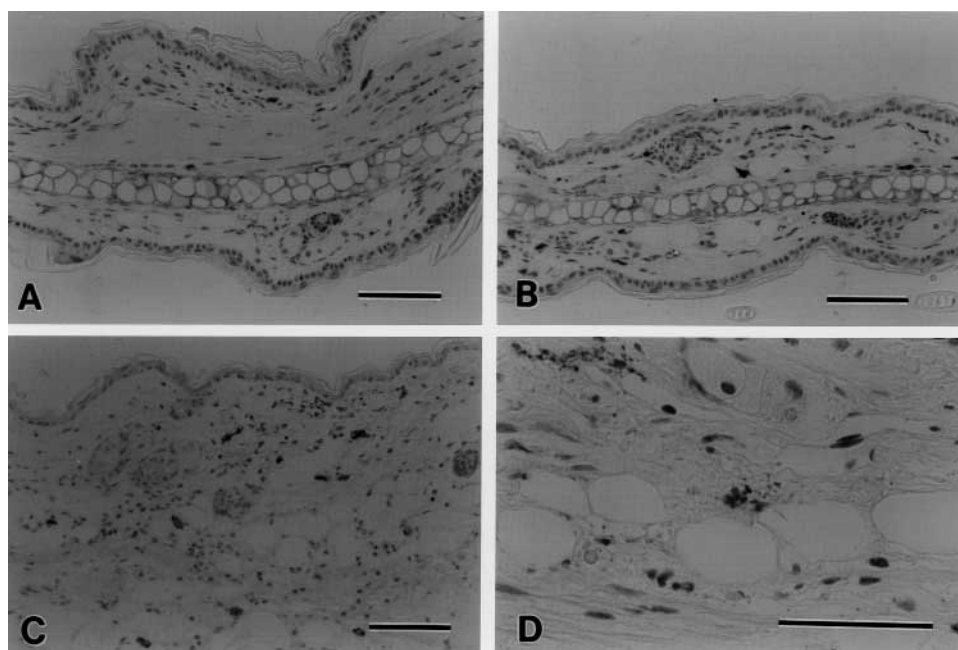


FIG 4. Immunohistochemistry for IL-6 in the ears of BALB/c mice after DNFB challenge. **A**, At 4 hours after acetone-olive oil (control) treatment. **B**, At 4 hours after DNFB challenge. **C** and **D**, At 8 hours after DNFB challenge. There is a significant increase of immunoreactive alcian blue-positive mast cells at 4 hours (**B**), which peaks at 8 hours (**C**). Extruded mast cell granules are positive for both alcian blue and for IL-6 immunoreactivity (**D**). Bar = 100 μ m (**A-C**) or 50 μ m (**D**). (Alcian blue and IL-6 double staining; original magnification, $\times 200$ [**A-C**] or $\times 400$ [**D**].)

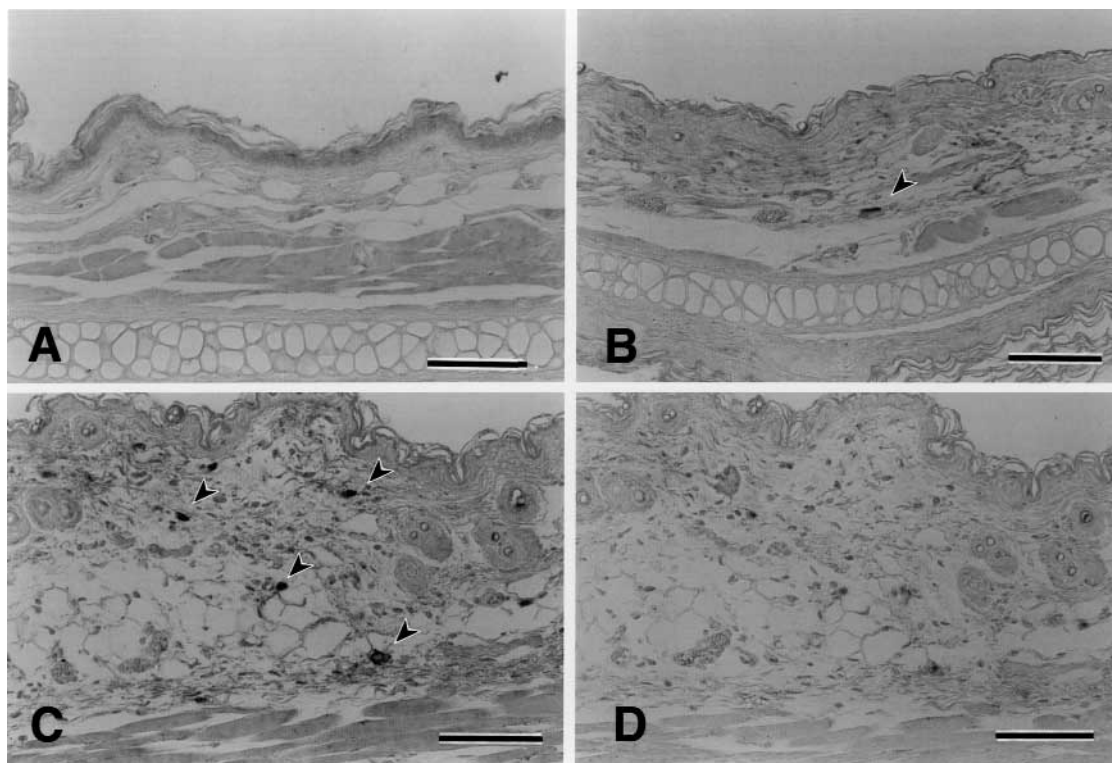


FIG 5. In situ hybridization of mouse IL-6 mRNA with the use of antisense oligonucleotide probes in the ears of BALB/c mice after DNFB challenge. Two antisense probes, MIL6A (bases 523-567) and MIL6B (bases 8-52), were used. **A**, No treatment; there are no reactive cells in the dermis. **B**, At 30 minutes after challenge; a few mast cells contain IL-6 mRNA. **C**, At 4 hours; there is a marked increase of IL-6 mRNA in mast cells from the edematous region. **D**, Competition test (same preparation as **C**); a 10-fold excess of sense probe was mixed with the hybridization solution and preincubated for 2 hours before hybridization. The positive reaction to MIL6A is markedly reduced. Bar = 100 μ m. (Alkaline phosphatase stain; original magnification, $\times 200$.)

hours, respectively (Fig 4, A-C). In the degranulated mast cells, extruded granules were also positive for IL-6 immunoreactivity (Fig 4, D). At 24 hours, the intensity of staining of the immunoreactive cells was weaker.

In acetone-olive oil-treated mice, the number of IL-6 immunoreactive mast cells was also increased (Fig 1, C) at 4 and 8 hours, but the staining intensity was lower than in antigen-challenged mice. Mild IL-6 immunoreactivity was seen in epidermal keratinocytes, sebaceous gland cells, and muscle cells, and a few fibroblasts or infiltrating mononuclear cells in the dermis after DNFB challenge, but not in neutrophils. Hyperplastic epidermal keratinocytes had the greatest staining intensity at 24 hours after the challenge.

In situ hybridization for IL-6 messenger RNA

We assessed IgE-dependent IL-6 gene expression in mast cells by using in situ hybridization to identify IL-6 mRNA expression. The expression of IL-6 mRNA was detected in a few resident mast cells in the dermis at 30 minutes after the DNFB challenge (Fig 5, A and B). At 4 hours, many mast cells at the edematous site and a few cells around it showed strong hybridization with both antisense oligonucleotide probes (MIL6A and MIL6B;

Fig 5, C). IL-6 mRNA expression by mast cells was detected until 8 hours after the challenge. Reaction with the MIL6A probe was inhibited by preabsorption with the sense probe (Fig 5, D), which demonstrated the specificity of the staining. The staining intensity was decreased 24 hours after the challenge. Weak expression of IL-6 mRNA was found in epidermal keratinocytes, sebaceous gland cells, muscle cells, and infiltrating neutrophils. On the other hand, acetone-olive oil-treated mice showed only slight IL-6 mRNA expression by dermal mast cells at 4 hours.

DISCUSSION

This study showed that AND and the expression of IL-6 mRNA and protein by mast cells within 4 hours after antigen challenge were important steps preceding the allergic cutaneous LPR in mice.

Allergic individuals, who are exposed to an appropriate antigen challenge, experience an IPR followed several hours later by the LPR. In recent years, investigators have focused on the LPR, because it is felt that this inflammatory response, which not only involves mediators but also mobilization of various types of cells,

reflects clinical disease more accurately than the less complex acute response.^{18,19}

Our previous study on IgE-mediated biphasic cutaneous responses used antiallergy drugs and soluble FcεRIα and WBB6F1-W/W^v mice to demonstrate the participation of mast cells in the IPR.⁶ In fact, a role of mast cells in the IPR has been confirmed by many investigators,^{20,21} but the role of mast cells in the LPR is still unknown. In the present study, we used the degranulation and the production of IL-6 as indicators of mast cell activation.

There is little information on the role of mast cell degranulation in the allergic cutaneous LPR in mice. Wershil et al²² reported that recruitment of neutrophils at 6 hours after IgE-mediated passive cutaneous anaphylaxis in mice was dependent on mast cell degranulation. In addition, Matsuda et al²³ reported that substance-P induced mast cell degranulation and the late infiltration of granulocytes in mouse skin. Both investigations indicated the importance of mast cell degranulation for late-phase accumulation of granulocytes in skin lesions but not for late-phase skin edema. Mast cells and basophils show 2 morphologic patterns of degranulation, termed *PMD* and *AND*, both in vitro and in vivo.^{24,25} These patterns are thought to represent separate points along a degranulation continuum. It has been reported that *PMD* generally occurs in the early phase of *AND* (within seconds) after stimulation by bacterial peptides and precedes the late phase, which occurs within minutes. *PMD* peaks in concert with maximum histamine release.²⁶ Tetradecanoyl phorbol acetate, which is a phorbol diester tumor promoter, stimulates *PMD* in human basophils that coincides with the slow release of histamine.²⁷ In our study, *PMD* occurred in the early period (until 1 hour) after the antigen challenge and preceded *AND* at 4 and 8 hours. Thus, *PMD* coincided with the IPR and *AND* with the LPR. The number of mast cells that exhibited *AND* peaked 8 hours after the challenge, and some mast cells were surrounded by neutrophils, which were phagocytizing the extruded mast cell granules. This suggests that mast cell degranulation and *AND* occurring at 4 to 8 hours after antigen challenge may play an important role in the onset of edema and inflammatory cell infiltration related to the LPR.

Mast cells are stimulated to synthesize IL-6 by the cross-linking of the FcεRI.^{28,29} In the present study, IL-6 mRNA was induced in mast cells as early as 30 minutes and peaked at 4 hours after the DNFB challenge, although there was an increase of mast cells immunoreactive for IL-6 protein from 4 to 8 hours after the challenge. There was no induction of IL-6 protein in mast cells within 1 hour of challenge. These results indicate that mast cell production of IL-6 protein in response to IgE-mediated stimulation coincided with the beginning of the LPR. The intensity of IL-6 immunostaining varied considerably, but immunoreactivity was observed in the cytoplasm and granules of mast cells. Mast cells that showed *AND* were only weakly immunoreactive for IL-6, although extruded granules were IL-6 positive, which suggests that the release of IL-6 protein in mast cell granules was a result of *AND*.

IL-6 is produced in vitro by many different skin cells, including keratinocytes, endothelial cells, and fibroblasts.³⁰ Mican et al³¹ reported that immunoreactive IL-6 was not only present in mast cells but also within keratinocytes in passive cutaneous anaphylaxis-induced mice. In the present study, keratinocytes, sebaceous gland cells, and muscle cells were positive for IL-6 from 4 hours after the antigen challenge. Although acetone-olive oil treatment (control) also caused the induction of IL-6 positivity in mast cells and other cells at 4 hours after the challenge, the change was weaker than in antigen-challenged mice, as was IL-6 mRNA induction. This result indicates that stimulation by painting the skin also induces IL-6 production in some cells. Because acetone-olive oil treatment produced no IPR or LPR, the physiologic effect of IL-6 induced in response to skin painting is unclear.

In conclusion, we demonstrated that IgE-mediated mast cell activation (indicated by *AND* and IL-6 expression) at 4 to 8 hours after antigen challenge is important for the development of the LPR.

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