

Expression of interleukin-4 and interleukin-5 mRNA in developing cutaneous late-phase reactions

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The stimuli for the accumulation and activation of eosinophils, which are prominent components of IgE-mediated allergic late-phase reactions (LPRs), are not defined. Messenger RNA for interleukin-5 has been found in lymphocytes present in biopsy specimens of skin obtained during LPR 24 hours after antigen challenge. However, it is not known whether interleukin-5 is present to attract or activate the eosinophils that accumulate during the first 6 hours after antigen challenge when cutaneous LPRs are developing. Using reverse transcription-polymerase chain reaction, we have found mRNA for interleukin-5 in biopsy specimens obtained during cutaneous LPR 6 hours after antigen challenge, but not in biopsy specimens obtained from control challenge sites nor in specimens from sites of antigen challenge of nonreactive subjects. We also found mRNA for interleukin-4 in these sites developing LPR. This pattern suggests that these cytokines may be important in eosinophil accumulation and activation during developing LPR. These findings are of considerable clinical relevance because the eosinophils in LPR are postulated to play major pathogenic roles in chronic allergic diseases. (J ALLERGY CLIN IMMUNOL 1995;96:92-6.)

Key words: Late-phase reactions, interleukin-4, interleukin-5, eosinophils, cytokines

IgE-mediated reactions have classically been considered to be immediate-type hypersensitivity reactions; however, the immediate reaction is followed by local inflammation, which peaks at 6 to 12 hours after contact with the allergen.^{1,2} This later response has been referred to as the late-phase reaction (LPR) and has been postulated to contribute to the pathogenesis of chronic allergic diseases.^{2,3} Several investigators have specifically implicated eosinophils, which accumulate prominently in LPR, as a major pathogenic mechanism. Although activated eosinophils elaborate the proinflammatory mediators leukotriene C₄ and platelet activating factor and release potentially

Abbreviations used

GM-CSF:	Granulocyte-macrophage colony-stimulating factor
IFN- γ :	Interferon- γ
IL:	Interleukin
LPR:	Late-phase reaction
RT-PCR:	Reverse transcription-polymerase chain reaction

toxic cationic proteins such as major basic protein,^{4,6} the nature of the stimuli for accumulation and activation of the eosinophils in LPR is unknown. Candidates for such stimuli include cytokines, such as interleukin (IL)-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF), which are known to attract or activate eosinophils.^{7,8} In situ hybridization studies have shown mRNA for IL-4, IL-5, and GM-CSF in biopsy specimens obtained during cutaneous LPR 24 hours after antigen challenge.⁹ Kay et al.⁹ have postulated that the sources of these cytokines are the CD4⁺ T cells, which they found to accumulate prominently near activated eosinophils in these 24-hour reaction sites.

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However, we have reported that eosinophils start accumulating within 2 hours of allergen challenge in sites of developing LPR.¹⁰ Many of these cells are activated and release granule components such as major basic protein and eosinophil-derived neurotoxin. It is not known whether cytokines are released early in the IgE-mediated reactions, which may lead to eosinophil activation when there is only modest accumulation of T lymphocytes. Therefore we have carried out analysis of such developing cutaneous LPRs for cytokine mRNA by using the reverse transcription-polymerase chain reaction (RT-PCR).

METHODS

Subjects

Five atopic human subjects (3 men and 2 women), sensitive to grass or ragweed extract (≤ 10 PNU/ml) (Greer Laboratories, Lenoir, N.C.) and one nonatopic subject, unreactive to these pollens, were studied after informed consent was obtained.

Skin testing and biopsies

Each subject received replicate intradermal injection of 0.03 ml of the relevant pollen extract and the buffered saline diluent. Six hours later, each site was anesthetized regionally with 2% lidocaine, and a 3 mm punch biopsy specimen was obtained from the intradermal injection site. Each specimen was coded and snap-frozen in liquid nitrogen until RNA extraction was performed.

RNA extraction, reverse transcription, and PCR

Total RNA was extracted from skin biopsy specimens with RNeasy B (Tel-Test, Inc., Friendswood, Texas), a modification of the guanidinium thiocyanate isolation method.¹¹ Because of the small amount of total RNA present in these biopsy specimens (typically 1 μ g), 0.1% vol/vol of glycogen (1 mg/ml; Boehringer Mannheim Berhing, San Diego, Calif.) was added as a carrier to ensure maximal recovery. After extraction, the RNA was reverse-transcribed as previously described.¹² Briefly, total RNA was heated to 65°C for 5 minutes and then cooled on ice. The RNA was then incubated at 37°C for 60 minutes in a cocktail containing reverse transcriptase (BRL/Gibco, Gaithersburg, Md.) deoxynucleotide triphosphates (Perkin-Elmer Cetus, Norwalk, Conn.), Oligo (dT) 16 (Sigma Chemical Co., St. Louis, Mo.), dithiothreitol (BRL), RNase inhibitor (RNasin, BRL), $MgCl_2$, KCl, and Tris buffer in a total volume of 20 μ l. Immediately after the incubation, the reverse transcriptase cocktail was divided into 5 aliquots for PCR amplification. PCR amplification of cytokine cDNA was accomplished with sense and antisense primers as previously described.¹³ To determine the integrity of the cDNA for each experimental sample, a separate control

amplification with β -actin primers was included in the PCR run. The PCR cocktail consisted of Tris buffer, 2.5 mmol/L $MgCl_2$, 1.5 mmol/L deoxynucleotide triphosphate, 500 ng each of the sense and antisense primers, 1.25 U of Taq polymerase (Promega Corp., Madison, Wis.), and H_2O in a total volume of 50 μ l. This PCR cocktail was amplified in a forced hot air thermocycler (BioOven II; BioTherm Corp., Fairfax, Va.) for 30 cycles in which a single cycle consisted of 92°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minutes. After amplification, a 12 μ l sample of the PCR cocktail was sized on an ethidium bromide-containing 3% Nusieve (FMC, Rockland, Maine): 1% agarose (Gibco/BRL) gel run at 90 V for 1 hour. The gel was visualized under UV light, and the size of amplification products was compared with a molecular weight marker (1 kb DNA ladder, 5616SA, Gibco/BRL) run in a parallel lane. The specific cytokine identity of PCR products was verified by size and by autoradiography with a phosphorous 32- α -adenosine triphosphate-labeled nested oligonucleotide probe.

RESULTS

With the RT-PCR approach described above, mRNA for IL-5 was found in coded biopsy specimens obtained from sites of developing LPR 6 hours after intradermal antigen injection in all five subjects tested (Fig. 1). In four of these sites, mRNA for IL-4 was also found (Fig. 2). Neither IL-5 nor IL-4 mRNA was found in biopsy specimens obtained 6 hours after intradermally injected diluent (Figs. 1 and 2), in biopsy specimens obtained 6 hours after antigen injection in a nonsensitive subject, or in normal skin.

In contrast, mRNA for IL-2 and interferon (IFN)- γ was expressed in biopsy specimens obtained from both antigen-induced LPR and diluent-injected sites in these subjects. We have previously found similar expression of mRNA for IL-2 and IFN- γ , but not IL-4 or IL-5, in normal skin from healthy subjects.¹³ It is unlikely that the detection of the mRNA of IL-2 or IFN- γ by RT-PCR in normal skin reflected the effects of contaminated reagents because concomitant RT-PCR analysis of cDNA from the Hut78 cells, a $CD4^+$ -transformed T-cell line, did not reveal expression of mRNA for IL-2 or IFN- γ .

The cellular source of the cytokine mRNA found here is not yet known. We found small numbers of T lymphocytes in unchallenged skin with modestly increased numbers in the sites of developing LPR 6 hours after intradermal antigen was injected (Fig. 3). Recent evidence suggests that human, like murine $CD4^+$ T cells, may be divided into T_{H1} and T_{H2} subsets.¹⁴⁻¹⁶ Activated T_{H1}

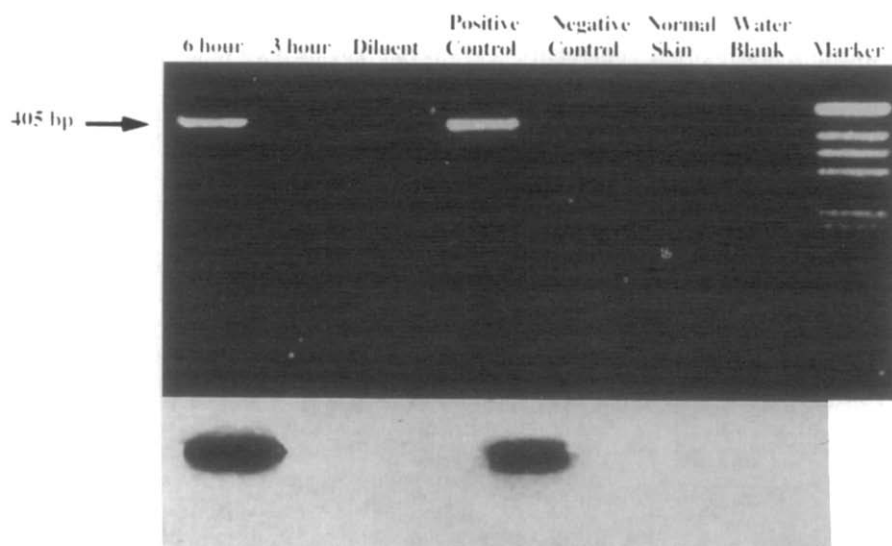


FIG. 1. Amplification of IL-5 mRNA from representative skin biopsy specimens after injection of antigen or diluent. *Top portion* of each photograph shows amplification productions on a 4% agarose gel; shown in the *bottom portion* of the photograph is the corresponding autoradiograph obtained after probing with a radiolabeled nested oligonucleotide probe for IL-5. *Lane 1*, Biopsy specimen obtained 6 hours after allergen challenge; *lane 2*, biopsy specimen obtained 3 hours after allergen challenge; *lane 3*, biopsy specimen from site injected with diluent only; *lane 4*, concanavalin A-stimulated (6 hours) peripheral blood mononuclear cells; *lane 5*, cDNA from Hut78 cell line; *lane 6*, biopsy specimen from volar region of the forearm of a normal control subject; *lane 7*, water blank; *lane 8*, molecular weight markers.

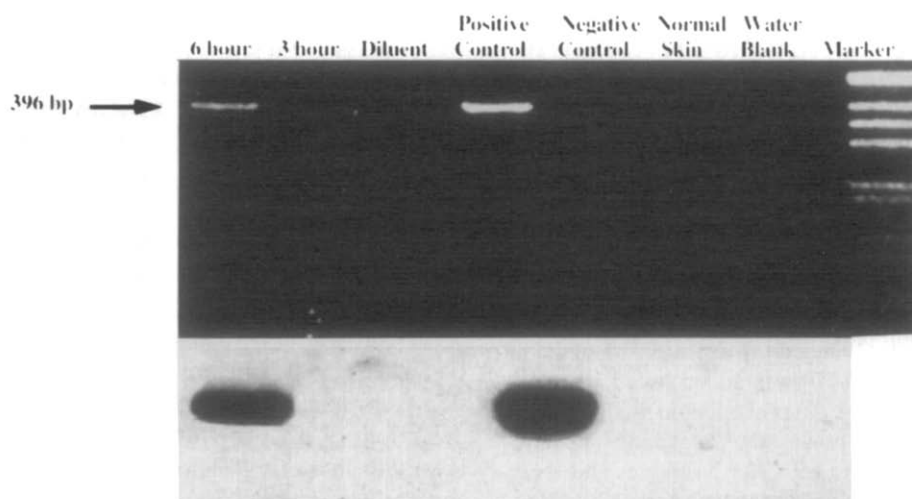


FIG. 2. Amplification of IL-4 mRNA from representative skin biopsy specimens after injection of antigen or diluent. See legend to Fig. 1 for explanation.

cells are thought to secrete predominantly IL-2 and IFN- γ , whereas activated T_{H2} cells secrete predominantly IL-4 and IL-5. Using in situ hybridization, Kay et al.⁹ have identified T cells as the major focus of mRNA for IL-3, IL-5, and GM-CSF in biopsy specimens obtained during LPR 24 hours

after intradermal antigen challenge. Although a lymphocytic infiltrate is present at 6 hours,^{1,17} we have not found a significant increase in the number of CD5⁺ cells at LPR sites compared with diluent-injected sites in biopsy specimens obtained 6 hours after injection. Therefore it is unclear whether a

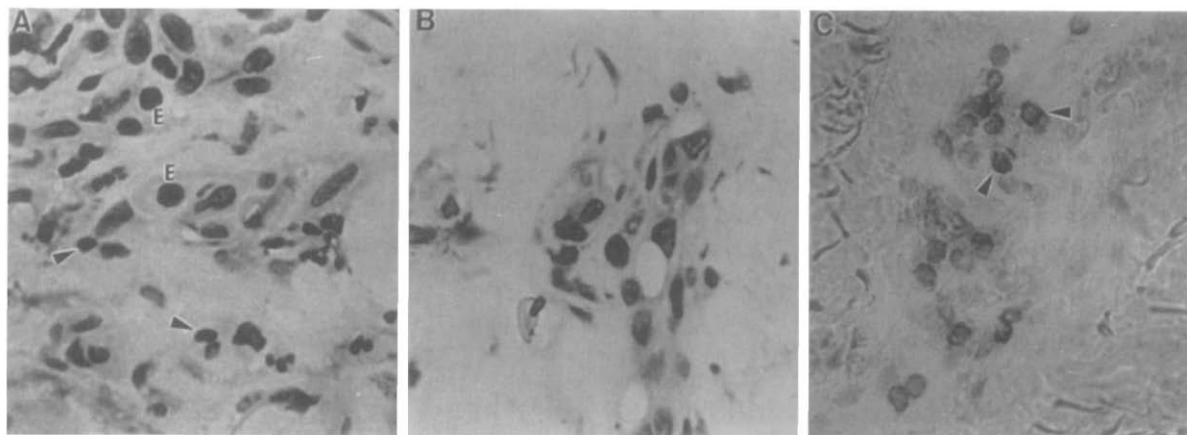


FIG. 3. Presence of T cells in developing LPRs. **A**, Biopsy specimen obtained during late-phase allergic reaction 6 hours after intradermal grass pollen injection. *E* indicates representative eosinophils as seen with hematoxylin and eosin staining; *arrows* denote representative mononuclear cells. One micron, JB-4 methacrylate-embedded tissue (total magnification, $\times 600$). **B**, Biopsy specimen obtained at site adjacent to **A** 6 hours after intradermal injection with diluent only. Note paucity of infiltrate as compared with **A**. One micron, JB-4 methacrylate-embedded tissue (total magnification, $\times 600$). **C**, Frozen section of a late-phase allergic reaction 6 hours after intradermal grass pollen injection in the same subject as in **A** and **B**. *Arrows* denote representative positive staining cells. Immunohistochemical staining for CD3⁺ T cells was performed with monoclonal anti-CD3 antibody and alkaline phosphatase–antialkaline phosphatase technique.

similar pattern of cytokine mRNA would be detected in T cells at that earlier time point.

DISCUSSION

Even though Kay et al.⁹ have found cytokine expression in T cells at 24 hours, it remains possible that cells other than the infiltrating T cells could be the source of the IL-4 and IL-5 mRNA observed in this study. Recent studies of both mice and human beings suggest that activated mast cells may contain one or both of these cytokines.^{18,19} Another possible source for the IL-5 mRNA in these developing LPRs is the infiltrating eosinophil population itself. As noted above, we have found accumulation of eosinophils and neutrophils, starting within the first several hours when LPRs are developing after antigen challenge.²⁰ Some of these cells are activated and releasing intracellular contents.^{10,21} Broide et al.²² have recently found that eosinophils present in bronchoalveolar lavage obtained 24 hours after inhalational antigen challenge contained mRNA for IL-5 and GM-CSF. If eosinophils have the capacity to secrete IL-5, the regulation of recruitment and activation of eosinophils in situ may be at least partially under autocrine control subsequent to an initiating signal. Such an initiating signal has not been identified; however, it is likely that it would be derived from

an endogenous cell, such as the mast cell or Langerhans' cell.

Although IL-5 may play a major role in the recruitment and activation of eosinophils, other agents released locally by endogeneous cutaneous cells may play important roles in the pathogenesis of LPR, particularly at early time points when large numbers of infiltrating, activated T cells are not present. In this regard, the role of other cytokines, such as IL-3, GM-CSF, and RANTES in LPRs, should certainly be considered. RANTES has recently been found to be a strong attractant of neutrophils and to enhance eosinophil chemoattraction. It may also activate such cells and attract CD4⁺ CD45RO⁺ "memory" T cells.^{23,24} Such a selective pattern of chemoattraction may account for the pattern of cellular infiltrates associated with LPR.²⁵ However, RANTES did not induce leukotriene C₄ production and caused the release of only small amounts of eosinophil cationic protein by eosinophils, again indicating that a network of cytokine interactions appears to be involved in the generation of LPRs.²³

The sources of the mRNA for IL-2 and IFN- γ that we found in both LPR and control sites are not known. However, because there are no known endogenous skin cells that secrete IL-2 or IFN- γ either spontaneously or when activated, the most

likely source is the small number of CD3⁺ T cells seen in the perivascular region of normal skin. Of note, these T cells also frequently express activation markers, such as CD25 and major histocompatibility complex class II antigens.²⁶ Because IL-2 mRNA is generally seen in cells before expression of these activation markers,²⁷ it is not surprising that mRNA for IL-2 and IFN- γ , products of activated T cells, is detectable in normal skin by RT-PCR.

The findings described here extend previous observations in an important way by indicating that mRNA for IL-4 and IL-5 appear during the developing stages of LPR, as well as at 24 hours when LPR has been established for some time. Thus cytokine generation likely plays important roles in the initiation and the perpetuation of LPRs. Although much has to be learned about the pathogenesis of the LPR, further investigation of the role of cytokines is indicated, particularly because of evidence pointing to a pathogenic role of the LPR in chronic allergic disorders of the skin and respiratory tract.^{2, 3, 28, 29}

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