The Kinetics of Allergen-induced Transcription of Messenger RNA for Monocyte Chemotactic Protein-3 and RANTES in the Skin of Human Atopic Subjects: Relationship to Eosinophil, T Cell, and Macrophage Recruitment

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Summary

The C-C chemokines RANTES and monocyte chemotactic protein-3 (MCP-3) are potent chemoattractants in vitro for eosinophils and other cell types associated with allergic reactions. We tested the hypothesis that the allergen-induced infiltration of eosinophils, T cells, and macrophages in the skin of atopic subjects is accompanied by the appearance of $mRNA^+$ cells for RANTES and MCP-3. Cryostat sections were obtained from skin biopsies from six subjects 6, 24, and 48 h after allergen challenge. Tissue was processed for immunocytochemistry (ICC) and for in situ hybridization using ^{35}S-labeled riboprobes for RANTES and MCP-3. In contrast to diluent controls, allergen provoked a significant increase in $mRNA^+$ cells for MCP-3, which peaked at 6 h and progressively declined at 24 and 48 h. This paralleled the kinetics of total (major basic protein positive $[MBP]^+$) and activated (cleaved form of eosinophil cationic protein $[EG2]^+$) eosinophil infiltration. The allergen-induced expression of cells $mRNA^+$ for RANTES was also clearly demonstrable at 6 h. However, the numbers were maximal at 24 h and declined slightly at the 48-h time point. The number of $mRNA^+$ cells for RANTES paralleled the kinetics of infiltration of $CD3^+$, $CD4^+$, and $CD8^+$ T cells whereas the number of $CD68^+$ macrophages was still increasing at 48 h. These data support the view that MCP-3 is involved in the regulation of the early eosinophil response to specific allergen, whereas RANTES may have more relevance to the later accumulation of T cells and macrophages.

The allergen-induced cutaneous late phase response (LPR)[1] is a useful model of atopic allergic inflammation (1, 2). By applying the techniques of immunocytochemistry (ICC) and *in situ* hybridization (ISH) to full thickness skin biopsies, the LPR was shown to be associated with the infiltration of $EG2^+$ eosinophils, $elastase^+$ neutrophils, $CD3^+$, $CD4^+$, and $CD8^+$ T cells, $UCHL1^+$ ($CD45RO^+$) memory cells, $CD68^+$ macrophages, as well as cells expressing mRNA for the Th2-type cytokines IL-4 and IL-5 (1–5). The precise mechanisms for the recruitment of eosinophils, T cells, and other inflammatory cells into allergen-injected sites are still unknown. However, present evidence suggests that this process involves selective cell adhesion, chemotaxis and prolonged survival of specific inflammatory cells consequent to the local release of cytokines and other mediators from IgE-sensitized mast cells and allergen-specific T cells (6–8).

The role of chemokines as specific chemotactic factors in allergic tissue reactions is of considerable current interest. Chemokines, also known as intercrines, comprise a superfamily of small peptides (8–14 kD) which can be divided into two subfamilies, based on the arrangement of the structural motif containing two cysteines, either separated or adjacent (C-X-C chemokines and C-C chemokines, respectively) (7). Chemokines of the C-X-C type include IL-8 and are predominantly chemotactic in vitro for neutrophils. In contrast, C-C chemokines attract T cell subsets and specific granulocytes other than neutrophils (7, 9–11). RANTES, a member of the C-C family, is chemotactic for eosinophils, basophils, monocyte/macrophages, and $CD4^+/UCHL1^+$ "memory" T cells (11–13). A more recently described C-C chemokine is monocyte chemotactic protein-3 (MCP-3) (14). MCP-3 is approximately equivalent, in term of potency, to RANTES in eosinophil and basophil chemotaxis (11, 15). The effect of MCP-3 on T cell locomotion has yet to be established. Since MCP-3 and RANTES also stimulate histamine release from human basophils and promote exocytosis of eosinophil granule proteins in vitro (11, 16, 17), they have the potential

[1] *Abbreviations used in this paper*: EG2, cleaved form of eosin cationic protein; ICC, immunocytochemistry; ISH, in situ hybridization; LPR, late-phase response; MBP, major basic protein; MCP-3, monocyte chemotactic protein-3; RANTES, regulated upon activation in normal T cells expressed and secreted; TEM, transendothelial migration.

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to play a special role in allergic inflammatory reactions. For these reasons, we hypothesized that exposure to a specific allergen induces transcription of mRNA for MCP-3 and RANTES in the skin of human atopic subjects and that the time course of appearance of mRNA$^+$ cells is related to that of the accumulation of eosinophils, T cells and macrophages.

Materials and Methods

Human Subjects. Six atopic subjects (two females and 4 males; mean age = 38.5 ± 2.8 yr old) were recruited from the Allergy Clinic of the Royal Brompton Hospital (London, UK) and from the staff of the National Heart & Lung Institute (London, UK) Inclusion criteria for the subjects were as follows: (*a*) age between 18 and 55 yr old; (*b*) history of seasonal and/or perennial allergic rhinitis and/or asthma; (*c*) absence of any other illness; (*d*) positive skin prick tests (weal diameter >5 mm) to Timothy grass pollen (*Phleum pratense*; Soluprick; ALK, Horsholm, Denmark) or house dust mite extract (ALK) in the presence of positive histamine and negative vehicle controls. Patients taking oral medication were not included in the study. The total IgE levels ranged from 101 to 1,780 IU/ml and all patients had positive RAST results to *Phleum pratense* (6.3–100 IU/ml).

Study Design. The study was approved by the Royal Brompton Hospital Ethics Committee and was performed with the patients' written informed consent. 30 BU of Timothy grass pollen extract (0.02 ml) were injected intradermally into three sites on the extensor aspect of the forearms of each subject. A fourth site was injected with a similar volume of diluent. Macroscopic responses were measured at 6, 24, and 48 h by evaluating skin induration by resistance to the movement of a sharpened pencil point with which the reaction was outlined. Permanent sticky tape records of the outlines of the responses at all time points were then made. One site injected with allergen was biopsied at 6, 24, or 48 h, immediately after measurement of the size of the reaction. The control site injected with diluent was biopsied at 24 h. In this way, each patient served as his/her own control. A 4-mm disposable biopsy punch was used to take biopsies after using 1% plain lidocaine for local anaesthesia.

Processing of Specimens. Tissue biopsies were immediately fixed in 4% paraformaldehyde (BDH Chemicals Ltd., Dagenham, UK) in PBS, pH 7.4, for 4 h, and washed with 15% sucrose (Sigma Chemical Co., Poole, UK) in PBS for 1 h twice, then snap frozen in isopentane (BDH Chemicals) cooled in liquid nitrogen. Cryostat sections (6 μm) were cut from biopsies, mounted on 0.1% poly-L-lysine-coated RNase-free slides, dried overnight at 37°C, then stored at −80°C.

ICC. The alkaline phosphatase anti-alkaline phosphatase technique was used to enumerate cells binding to monoclonal antibodies against human T cells (CD3, Becton Dickinson, Cowley, Oxon, UK), helper T cells (CD4, Becton Dickinson), cytotoxic T cells (CD8, Becton Dickinson), total eosinophils (BMK-13, [18]), activated eosinophils (EG2; Pharmacia, Uppsala, Sweden), and macrophages (CD68, Dako, High Wycombe, U.K.). The technique was performed as described previously (1–3).

ISH. cDNA for human RANTES and MCP-3 were kind gifts from Dr. P. Nelson (Department of Pediatrics, Stanford University, Stanford, CA) and Dr. G. Opdenakker (Laboratory of Molecular Medicine, Rega Institute, University of Leuven, Belgium), respectively (10, 11). Radiolabeled (^{35}S; Amersham International, Amersham, UK) riboprobes (both sense and antisense) were prepared as before (4–5). ISH was performed as previously described (4–5) with some modifications. Briefly, cryostat sections were permeabilized by immersion in 0.3% Triton X-100 in PBS for 10 min. After a brief wash in PBS, sections were further permeabilized by exposure to proteinase K (Promega, Southampton, UK) solution (1 μg/ml in 20 mM Tris-HCl and 1 mM EDTA, pH 7.2) for 30 min at 37°C, the activity of which was then terminated by immersion in 4% paraformaldehyde/PBS for 5 min. To inhibit nonspecific binding of ^{35}S-labeled probes, sections were treated with 10 mM iodoacetamide and *N*-ethyl-malemide (Sigma) for 30 min at 37°C and then 0.5% acetic anhydride and 0.1 M triethanolamine for 10 min before air drying. Sections were hybridized with ^{35}S-labeled riboprobes (either antisense or sense at 10^6 cpm per section) in hybridization buffer containing 100 mM DTT (Promega) at 55°C overnight. Slides were then washed twice in 50% formamide/2× SSC containing 10 mM DTT at 50°C for 20 min, incubated with 20 μg/ml of RNase A (Sigma) in 2× SSC for 30 min at 37°C to remove any unhybridized RNA, and washed in 2× SSC (65°C, 20 min) followed by 0.1× SSC (65°C, 20 min). After dehydration, sections were immersed in K-5 emulsion (Ilford, Basildon, UK) and exposed for 14 d at 4°C. The autoradiographs were developed in developing solution (D-19; Eastman Kodak Co., Rochester, NY), fixed with Hypam (Ilford), and counterstained with hematoxylin. For negative controls, slides were hybridized to sense probes or pretreated with RNase A prior to hybridization with antisense probes (4,5).

Quantitation. For both ICC and ISH, slides were counted blind in a coded random order by the two observers. Hybrids between chemokine mRNA and riboprobes were localized as dense collections of silver grains overlying individual cells. Since it was not possible to count individual silver grains, cells expressing chemokine-specific mRNA were quantified in terms of the numbers of cells with overlying silver grains. When positive cells were in close proximity, their numbers were determined by visualizing individual nuclei using dark-field illumination. Positive cells were counted with an eyepiece graticule at a magnification of 200. For each biopsy, at least two sections were stained for each antibody (ICC) or hybridized with each riboprobe (ISH), from which four to six fields were counted. Results are expressed as the numbers of positive cells per field (0.202 mm^2). The mean coefficient of variation of the cell counts did not exceed 5%.

Analysis. Data were analyzed using a statistical package (Minitab Release 7, Minitab Inc., State College, PA). Comparisons within the different time points for each cellular marker or each antisense riboprobe were performed by Friedman's test. For all tests, $P < 0.05$ was considered significant.

Results

All subjects exhibited cutaneous LPR after allergen but not diluent control challenge ($P < 0.02$). The mean size (mm ± SEM) of the LPR was 75.7 ± 10.5 (6 h), 71.8 ± 15.3 (24 h), and 68.5 ± 16.5 (48 h).

Using Friedman's test, which compares diluent with all three allergen-challenged time points (i.e. 6, 24, and 48 h), there were significant increases in the numbers of CD3$^+$ ($P = 0.002$), CD4$^+$ ($P = 0.004$), CD8$^+$ ($P = 0.014$) T cells, eosinophils (MBP$^+$ and EG2$^+$, $P < 0.001$), and CD68$^+$ macrophages ($P = 0.004$). The mean numbers of CD3$^+$, CD4$^+$, and CD8$^+$ cells were maximal at 24 h after allergen challenge and subsequently declined, although they remained elevated compared to those in diluent-challenged sites up to

48 h. In contrast, eosinophil numbers were maximal at the 6 h time point, then declined at 24 h and at 48 h, although they were still elevated at these time points when compared to those in diluent-challenged sites. The numbers of CD68+ macrophages infiltrating the allergen-challenged sites continued to be elevated for up to 48 h after challenge.

Friedman's test also showed significantly increased numbers of cells expressing chemokine mRNA for RANTES and MCP-3 (P = 0.005 and 0.003, respectively; Fig. 1). The numbers of cells expressing RANTES mRNA were maximal at 24 h after allergen challenge, whereas the peak expression for MCP-3 was at 6 h. No significant correlation between the number of cells expressing MCP-3 or RANTES and the number of infiltrating inflammatory cells was observed. At diluent-challenged sites, very occasional hybridization signals were observed for RANTES and MCP-3. No hybridization signals were observed in the sections pretreated with RNase A or the sections treated with the sense probes (Fig. 2). These observations further confirm that the hybridization signals observed represent specific binding and between target mRNA and antisense riboprobes.

Chemokine mRNA-positive cells were generally located within areas of inflammatory infiltrate at the upper part of the dermis as well as in the deep reticular dermis (Fig. 2).

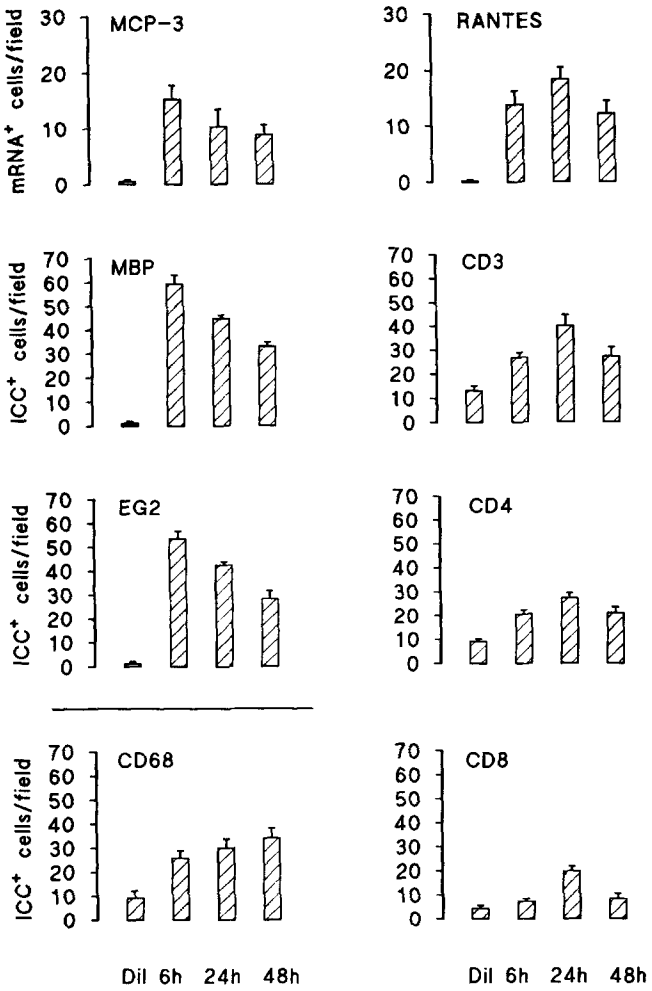


Figure 1. Time course of appearance of mRNA+ cells for MCP-3 and RANTES, eosinophils (MBP+ and EG2+ cells), T cells (CD3+, CD4+ and CD8+) and macrophages (CD68+) into allergen-challenged skin sites in atopic subjects (n = 6). Diluent-challenged sites (*Dil*) were used as controls. The results are expressed as the numbers of positive cells (mean ± SEM) per field (0.202 mm^2) of skin biopsies. Significant differences (diluent, 6-, 24-, and 48-h allergen-challenge time points, Friedman's test) were for MCP-3 (P = 0.003), RANTES (P = 0.005), MBP and EG2 (P <0.001), CD3 (P = 0.002), CD68 and CD4 (P = 0.004) and CD8 (P = 0.014).

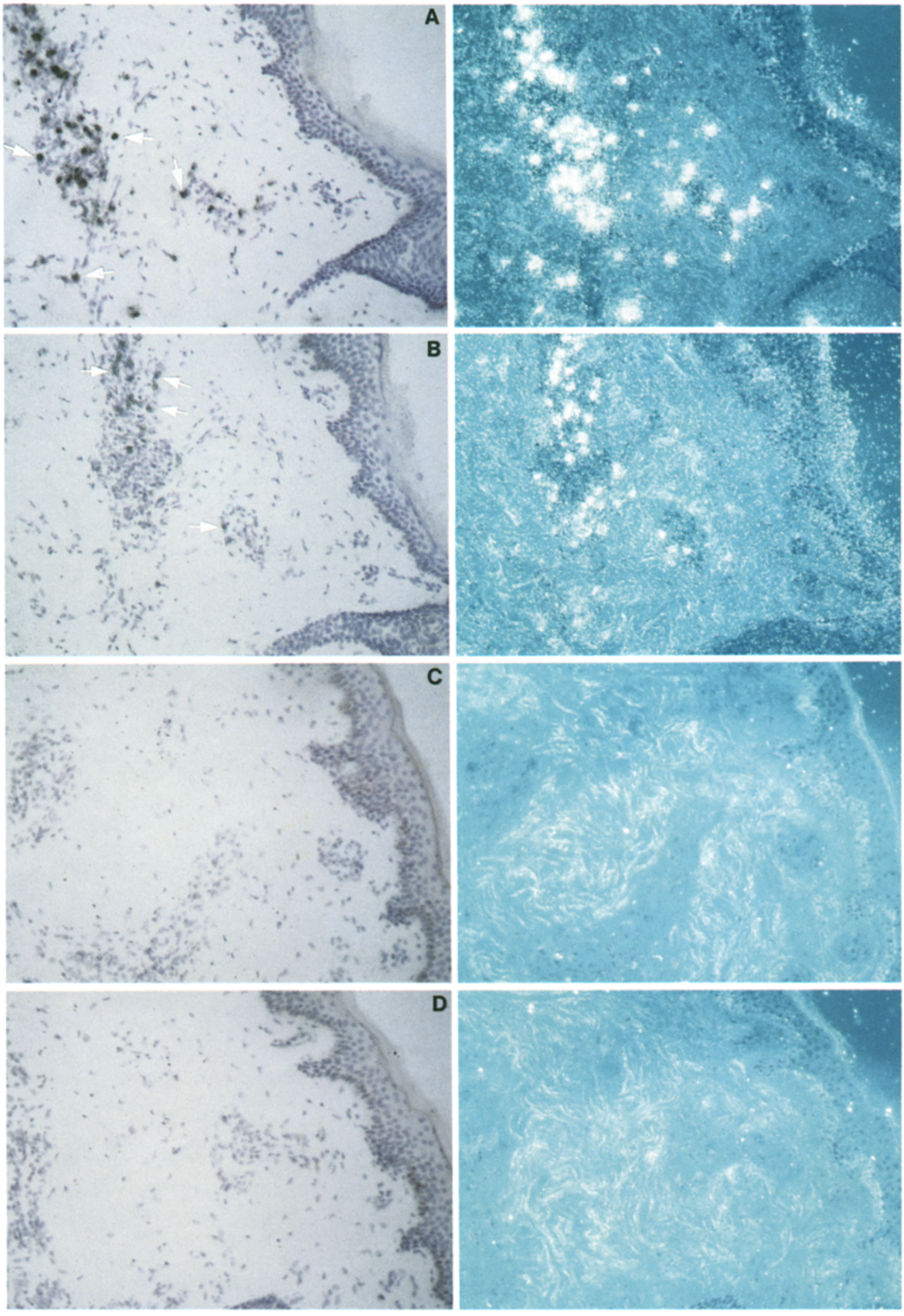
Discussion

The novelty of this study is (*a*) the demonstration of MCP-3 and RANTES mRNA-positive cells in human tissue and (*b*) their induction by specific antigen in sensitized atopic subjects. Furthermore, we observed an early peak (6 h) in MCP-3 and a later peak (24 h) in RANTES mRNA-positive cell numbers. Although there were no significant correlations between the numbers of chemokine mRNA-positive cells and the various specific cell types, the time courses of the expression of mRNA encoding MCP-3 and RANTES were similar to the time courses of accumulation of eosinophils and T cells, respectively. While these observations give no definite information as to the cellular source of the chemokine mRNA, they are compatible with the hypotheses that MCP-3 and RANTES are important in eosinophil and T cell recruitment (11–16), or that these cells are a possible source of these chemokines.

CC chemokines such as MCP-3 and RANTES may act in concert with other cytokines, such as IL-4 and IL-5, which are also implicated in recruiting eosinophils into sites of allergic inflammation (6, 7, 11). For example, Ebisawa et al. showed that RANTES was an effective inducer of eosinophil transendothelial migration (TEM) in vitro, and that preexposure of eosinophils to IL-5 substantially potentiated the TEM response to RANTES (19). Furthermore, when human RANTES was injected into the skin of dogs, this induced local accumulation of eosinophils, monocytes, and lymphocytes, which was maximal at 16–24 h after injection (20). All these observations indicate that MCP-3 and RANTES may play an important role in local eosinophil recruitment.

It was previously demonstrated that the majority of CD4+ T cells infiltrating the sites of allergen-induced cutaneous LPR were of the "memory" (CD45RO+) phenotype (3). The present findings are consistent with a role for locally released RANTES in recruiting cells of this phenotype (13).

The cell type(s) responsible for positive hybridization signals for RANTES and MCP-3 will be the subject of further studies. However, it is known that MCP-3 is mainly produced by monocytes (7), whereas many cell types synthesize RANTES. These include T cells, epithelial cells, platelets, fibroblasts, macrophages, and endothelial cells (12, 21–23). It is also possible that the cell types expressing RANTES may differ (*a*) in various types of inflammation and (*b*) during the time course of a specific allergic inflammatory response such as the allergen-induced LPR.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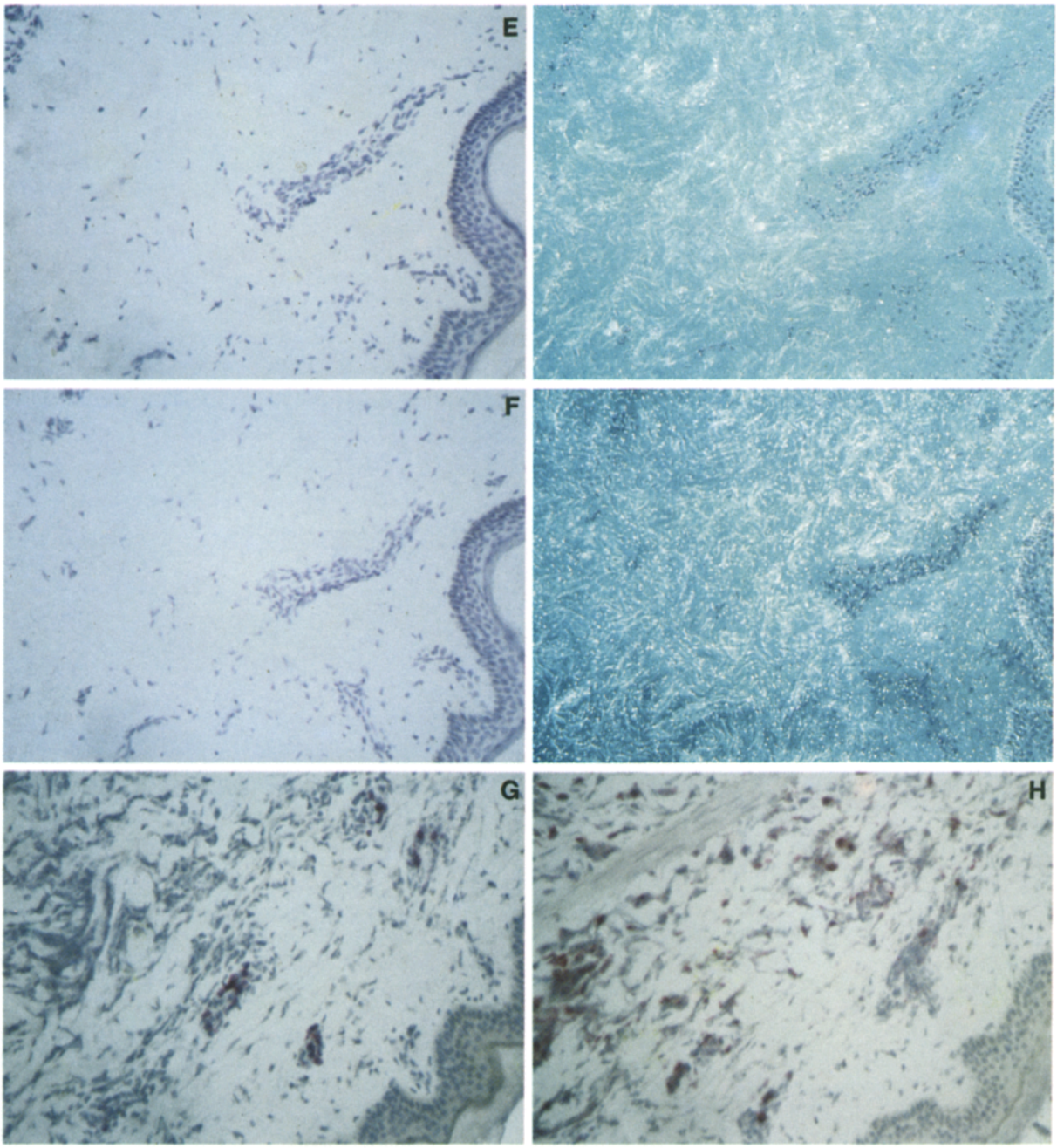

Figure 2. (*A–H*) Serial cryostat sections of a representative skin biopsy from an atopic subject 24 h after allergen challenge and diluent challenge. (*A*) In situ hybridization with ^{35}S-labeled antisense riboprobe for RANTES. Several RANTES mRNA$^+$ cells are observed (*left, arrowed*). The same section shown in dark field (*right*). (*B*) mRNA$^+$ cells for MCP-3 from the same biopsy (bright and dark field). Note that fewer cells were mRNA-positive for MCP-3 than for RANTES 24 h after allergen challenge. No positive hybridization signals were observed when the sections were hybridized with ^{35}S-labeled sense riboprobes for RANTES (*C*, bright and dark field) or MCP-3 (*D*, bright and dark field). No positive signals were found in the diluent challenge sites by use of ^{35}S-labeled antisense riboprobes for RANTES (*E*, bright and dark field) or MCP-3 (*F*, bright and dark field). (*G* and *H*) The infiltration of CD4$^+$ T cells and EG2$^+$ eosinophils, respectively, 6 h after allergen challenge.

As previously stated, RANTES and MCP-3 have also been found to be chemotactic for basophils (11, 15). However, a specific phenotypic marker for this cell type is not currently available for immunocytochemical studies. At the present time, there is no evidence that C-C chemokines enhance the survival of eosinophils and basophils in vitro. It is, therefore, reasonable to hypothesize that C-C chemokines play a role in TEM of T cells, basophils, and eosinophils, whereas TH2-type cytokines (e.g., IL-3, IL-4, IL-5, and GM-CSF) (4, 5, 8) cooperate with CC chemokines to prime, activate, and enhance survival of these cell types. Thus, all these elements have the potential to amplify atopic allergic reactions in humans

(11). Therefore, the inhibition of C-C chemokines and Th2-type cytokines may have therapeutic relevance in the treatment of atopic disease.

In conclusion, the demonstration of increased numbers of cells expressing C-C chemokine mRNA for RANTES and MCP-3 with different kinetics suggests that these chemokines may participate in the recruitment of inflammatory cells in atopic allergic inflammation.

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