*Brief Definitive Report*

# Production of the RANTES Chemokine in Delayed-type Hypersensitivity Reactions: Involvement of Macrophages and Endothelial Cells

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## Summary

To understand the selective accumulation of memory T helper lymphocytes and of macrophages in delayed-type hypersensitivity (DTH) granulomas, we studied the in situ production of RANTES, a chemokine initially characterized on the basis of its in vitro chemotactic properties for each of these cell populations. RANTES gene expression was studied by in situ hybridization in 15 human lymph nodes presenting typical DTH lesions related to either sarcoidosis or tuberculosis. A positive signal was detected in all cases. Labeling was specific for the DTH lesions, as very few if any positive cells were detected in the normal residual lymphoid tissue surrounding them or in reactive lymph nodes involved in a B lymphocyte response. RANTES gene expression was associated with the production of the protein, which was detected by immunochemistry in DTH lymph nodes. The morphological characteristics and distribution of positive cells in in situ hybridization and immunochemical experiments indicated that macrophages and endothelial cells, two cell populations not previously reported to produce RANTES, contributed to its production in DTH reactions. The ability of macrophages and endothelial cells to produce RANTES was confirmed by in vitro studies with alveolar macrophages and umbilical vein endothelial cells. In view of the chemotactic properties of RANTES for a limited range of cell populations, these results suggest that RANTES production in DTH granulomas may play a role in the selective accumulation of macrophages and memory T helper lymphocytes characterizing this type of cell-mediated immune reaction, and that macrophages and endothelial cells are involved in this production.

Recruitment and in situ accumulation of immune cells during an immune reaction is tightly regulated, and the nature of the cell populations homing to immunologically active sites varies according to the type of immune response. During delayed-type hypersensitivity (DTH) reactions, two cell populations are specifically recruited at the site of the lesion: T lymphocytes of the helper, memory subset ($CD4^+CD45RO^+$) and cells of the monocytic/macrophage lineage. Their in situ accumulation leads to the formation of a granuloma, which is the histological hallmark of DTH reactions. Other cell populations such as $CD4^+CD45RA^+$ T lymphocytes, $CD8^+$ T lymphocytes, and B lymphocytes are mostly confined to the residual lymphoid tissue surrounding active granulomas (reviewed in 1–4). This differing cell distribution outlines the selectivity of the homing process in the constitution of DTH granulomas.

A mechanism that may account for the accumulation of discrete cell subsets is the local production of specific chemotactic cytokines, or chemokines (reviewed in 5, 6). One of them, RANTES, was shown in vitro to be preferentially chemoattractive for human $CD4^+$ memory T lymphocytes and monocytes, whereas it displays no significant chemotactic property for $CD4^+$ naive T lymphocytes, $CD8^+$ cytotoxic T lymphocytes, B lymphocytes, or neutrophils (7).

The populations of immune cells sensitive to the chemoattracting properties of RANTES thus appear to be similar to those that are detected in DTH granulomas. This led us to ask whether in situ production of RANTES could ex-

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plain the pattern of immune cells accumulating in DTH reactions. We report that RANTES is indeed specifically expressed in DTH granulomas, and that in this condition cell populations not previously reported to produce RANTES contribute to its synthesis.

## Materials and Methods

*Tissues and Cells.* All tissues were processed as previously described (8). They included 15 lymph nodes displaying abundant granulomas typical of DTH reactions, related to sarcoidosis in eight cases and tuberculosis in seven cases, and 6 lymph nodes exhibiting follicular hyperplasia of unknown origin.

Alveolar cells were obtained by bronchoalveolar lavage (BAL) of two healthy human volunteers. Adherent cells were suspended at $10^6$ cells/ml in RPMI 1640 supplemented with 10% AB serum and cultured in the presence of IFN-$\gamma$ (500 U/ml) for 4 d. They were then centrifuged at 800 *g* for 10 min using a cytospin, air-dried for 2 h, fixed in acetone for 5 min, and stored at −80°C until used. More than 95% of these BAL cells expressed either CD68 or HLA-DR, as assessed by immunochemistry and flow cytometry, respectively.

Human umbilical vein endothelial cells (HUVEC) were extracted by collagenase treatment of human umbilical veins. They were cultured ($5 \times 10^4$ per $cm^2$) in MCDB 107 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum, 90 $\mu$g/ml heparin (Sigma Chemical Co.), and 40 $\mu$g/ml endothelial cell growth supplements (ECGS; Sigma Chemical Co.). More than 90% HUVEC were labeled with a polyclonal antifactor VIII antibody (Dakopatts, Glostrup, Denmark). Before stimulation they were washed and cultured during 24 h in fresh medium without heparin and ECGS. They were then placed in fresh medium without heparin and ECGS and stimulated or not with TNF-$\alpha$ (200 U/ml) and IFN-$\gamma$ (200 U/ml). After 24 h, supernatants and cells were recovered as described above.

*In Situ Hybridization.* In situ hybridization experiments were performed on frozen tissue sections as previously described (8). The RANTES-specific probe was constructed by cloning a 411-bp EcoRI–ApaI fragment of the human RANTES cDNA (for sequence, see reference 9) across the EcoRI–ApaI restriction sites of the Bluescript plasmid (Stratagene, La Jolla, CA). The antisense probe was obtained by linearizing the resulting plasmid with EcoRI and synthesizing a cRNA from the T3 promoter. The sense probe was obtained by linearizing the plasmid with ApaI and using the T7 promoter. In situ hybridization for IL-1$\beta$ and IFN-$\gamma$ was as previously described (10). For each patient, four to eight tissue sections were analyzed.

*Immunohistochemical Analysis.* The RANTES mAb (an IgG2a) was used at a final dilution of 0.9 $\mu$g/ml. As a control, a mAb of the same isotype, the F39-20 mAb (a gift from M. A. Petit, INSERM U131) (11), was used at the same concentration. CD68 mAb was from Behring (KiM7; Rueil-Malmaison, France) and CD3 mAb was from Becton-Dickinson (Pont-de-Clay, France). Immunohistochemical analysis was performed using an avidin-biotin technique according to manufacturer's recommendations (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA) except that 50% (vol/vol) normal human serum was added in the first blocking step.

*RANTES ELISA.* RANTES concentrations were measured by a sandwich ELISA, the details of which are to be published elsewhere (Sadick, M., and T. Schall, manuscript in preparation). Briefly, the assay employs two anti-RANTES mAbs that recognize different, noncompeting determinants. A standard curve was generated as described (12). The specificity of the assay was tested with other soluble proteins, including other chemokines of both the C-X-C (IL-8, melanocyte growth stimulatory activity) and C-C (macrophage inflammatory protein [MIP]-1$\alpha$, MIP-1$\beta$, monocyte chemotactic protein [MCP]-1) classes, none of which were shown to cross react in the assay.

## Results

*Expression of the RANTES Gene in DTH Reactions.* Expression of the RANTES gene was studied by in situ hybridization in 15 lymph nodes displaying morphological changes typical of granulomatous DTH reactions, related to sarcoidosis in eight cases and to tuberculosis in the seven other cases. Similar results were obtained for both conditions.

Positive cells were evidenced in all cases using the RANTES antisense probe (Fig. 1 *A*). They were abundant in most cases. No positive cells were detected in parallel experiments in which the RANTES sense probe was used as a control (Fig. 1 *B*). Cells expressing the RANTES gene were mostly located inside granulomas, and to a lesser extent in the inflammatory reaction surrounding granulomas. In contrast, they were absent or very rare in the normal residual lymphoid tissue of the lymph node.

Six lymph nodes exhibiting a follicular hyperplasia were tested in parallel. Cells containing RANTES mRNA were completely absent in four cases and exceptionally detected (less than two positive cells per tissue section) in the two other cases (data not shown).

*Production of the RANTES Protein in DTH Reactions.* Cells containing RANTES mRNA do not always produce the protein (13). We therefore analyzed in DTH reactions production of the RANTES protein using a specific mAb. Cells labeled with the anti-RANTES mAb were detected in 14 of the 15 DTH lymph nodes. They were mainly located inside granulomas (Fig. 1 *C*). Scattered cells were also present in the perigranulomatous inflammatory reaction, whereas they were either rare or absent in the normal lymphoid tissue. No signal was observed with a control mAb of the same isotype (Fig. 1 *D*).

Granulomas were usually diffusely stained in in situ hybridization and immunohistochemical experiments, although a peripheral pattern of in situ message expression and protein detection was seen in few cases. Thus, both approaches gave concordant results indicating local and specific production of the RANTES chemokine in DTH reactions.

*Characterization of RANTES-producing Cells in DTH Reactions.* Characteristics of the pattern of RANTES mRNA expression suggested that most positive cells in granulomas belonged to the macrophage rather than to the T lymphocyte lineage. Indeed, this pattern was identical to that obtained with an IL-1$\beta$ specific probe, a monokine previously shown to be strongly expressed by macrophages in DTH reactions (see reference 14), whereas it was quite different from that of INF-$\gamma$, which was detected in isolated and smaller cells corresponding to activated T lymphocytes (14). Immunochemical analysis with the anti-RANTES mAb showed that large cells with processes and epitheloid cells were stained. This pattern of labeling was similar to that of $CD68^+$ mac-

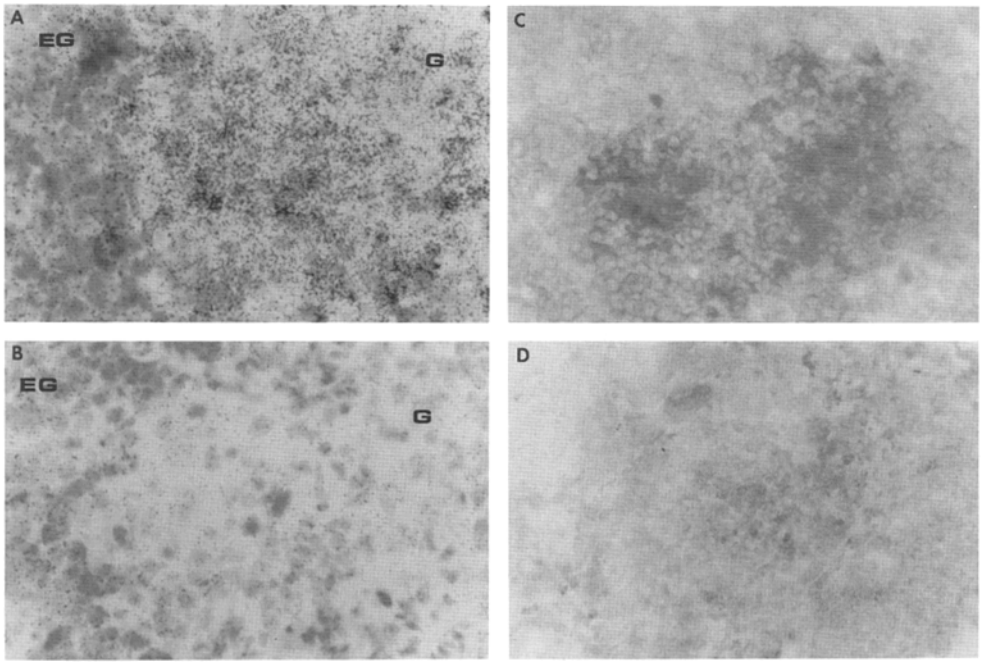


**Figure 1.** RANTES production in DTH granulomas. Production of RANTES was studied in 15 lymph nodes with DTH granulomas. In situ hybridization experiments were performed with an antisense (*A*) or a sense (*B*) probe specific for RANTES gene. Immunochemistry was performed with an anti-RANTES mAb (*C*) or with a control mAb (*D*). Original magnification: ×400 in *A* and *B*; ×200 in *C* and *D*. *G*, DTH granulomas; *EG*, extra-granulomatous lymphoid tissue.

rophages but clearly different from that of $CD3^+$ T cells (Fig. 2). Although the presence of RANTES-producing T lymphocytes cannot be formally ruled out based on morphological criteria, such cells if present should be much less abundant than RANTES-producing macrophages.

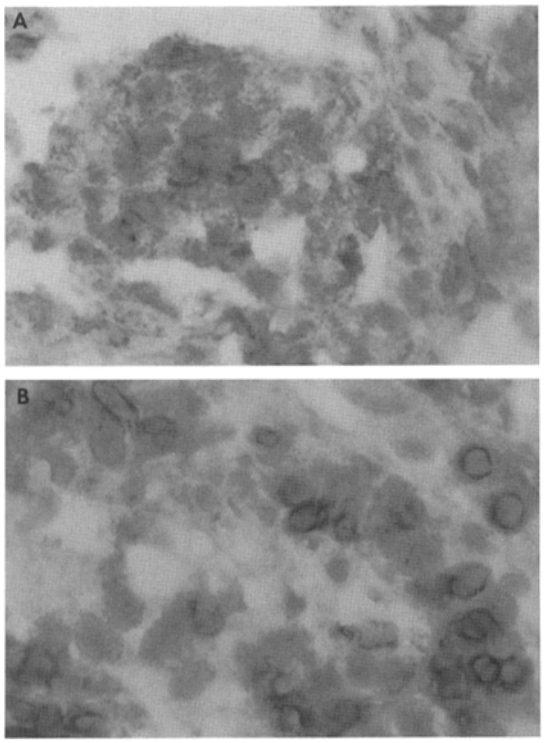


**Figure 2.** Characteristics of granulomatous cells. The morphology and distribution of cell populations in DTH granulomas were studied by immunochemistry. (*A*) Labeling of macrophages using a CD68 mAb; (*B*) labeling of T lymphocytes with a CD3 mAb. Original magnification: ×400.

In addition to macrophages, endothelial cells also expressed the RANTES gene. Indeed, cells whose morphology and location was consistent with that of endothelial cells were found to contain both RANTES mRNA and protein (Fig. 3). These RANTES-producing endothelial cells were found in the inflammatory reaction surrounding granulomas.

*In Vitro Production of RANTES by Macrophages and Endothelial Cells.* Neither macrophages nor endothelial cells have previously been reported to produce RANTES. To confirm our in vivo findings we tested whether these cell populations can synthesize the chemokine in vitro.

Production of RANTES by BAL macrophages was studied. Expression of the RANTES gene was not detected by in situ hybridization in freshly isolated cells. In contrast, >60% of cells were labeled with the RANTES antisense probe after 4 d of culture in the presence of IFN-$\gamma$ (Fig. 4). The supernatant of these 4-d cultures contained 770 pg/ml of RANTES.

HUVEC were also tested for RANTES gene expression. In the absence of stimulation, HUVEC were not labeled with the antisense probe, and their 24-h supernatant contained less than 200 pg/ml of RANTES. In contrast, more than 50% of cells stimulated with TNF-$\alpha$ and IFN-$\gamma$ expressed the RANTES gene in in situ hybridization experiments, and their supernatant contained 2,260 pg/ml of RANTES. Therefore, both macrophages and endothelial cells can produce RANTES upon stimulation.

## Discussion

This study shows that the RANTES gene is strongly expressed in lymphoid tissues involved in granulomatous DTH reactions. The production of RANTES appears to be specific to this kind of immune reaction, as virtually no cell expressed the RANTES gene in lymph nodes involved in a humoral

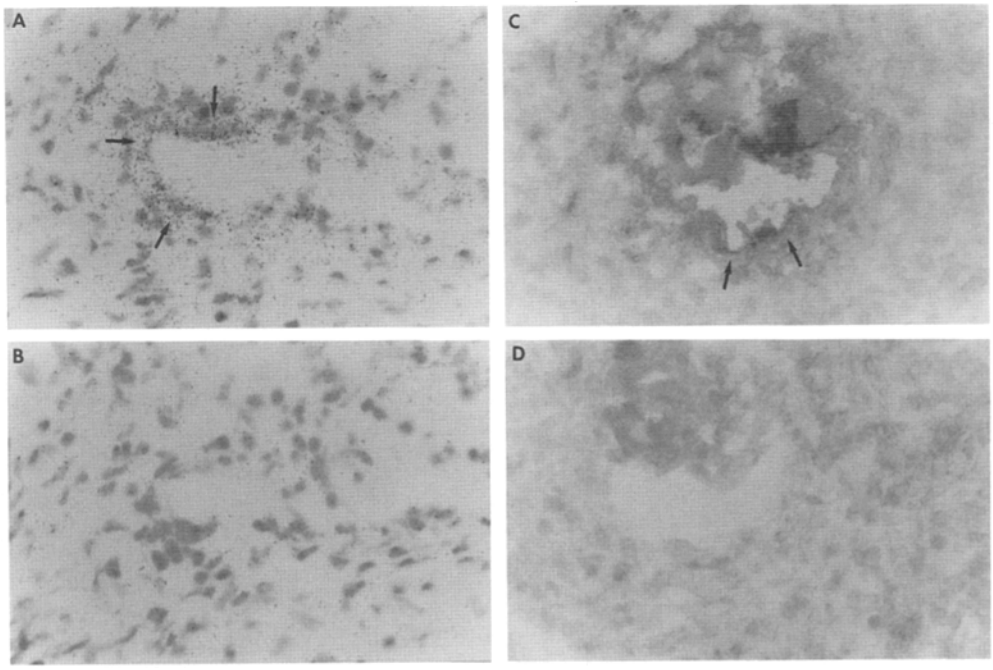


**Figure 3.** Production of RANTES by endothelium in perigranulomatous vessels. Lymph nodes with DTH granulomas were analyzed for the presence of cells with endothelial morphology and producing RANTES. In situ hybridization experiments were performed with an antisense (*A*) or a sense (*B*) RANTES probe. Immunochemistry was performed with an anti-RANTES mAb (*C*) or a control mAb (*D*). Original magnification: ×400 in *A* and *B*; ×250 in *C* and *D*. (*Arrows*) RANTES-producing cells.

immune response. The pattern of RANTES production was indistinguishable in cases of tuberculosis and sarcoidosis, indicating that the nature of the immune reaction in process, DTH, was more important than the triggering agent itself for induction of RANTES gene expression. In vivo expression of RANTES gene has been reported in arteriosclerosis (15), indicating that the spectrum of conditions in which RANTES is produced in humans is not restricted to DTH granulomas.

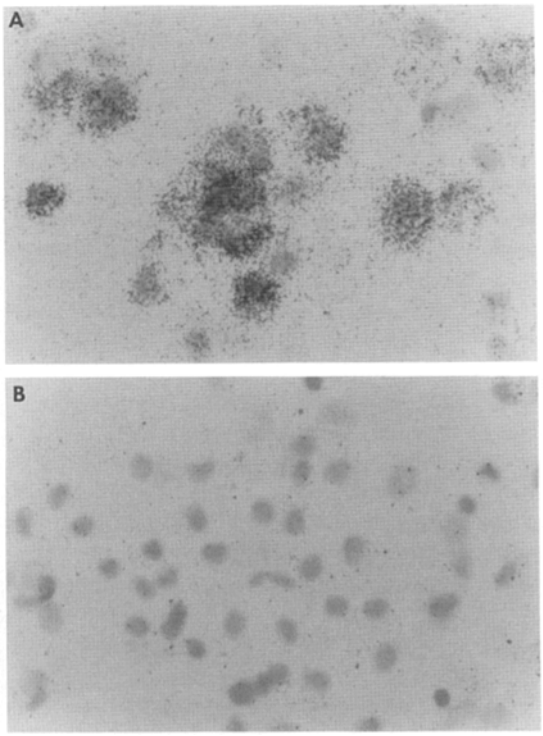


**Figure 4.** RANTES gene expression by normal macrophages. Human macrophages were collected by BAL and cultured for 4 d in the presence of IFN-γ (500 U/ml). Expression of the RANTES gene was then determined by in situ hybridization with an antisense (*A*) or a sense (*B*) probe. Original magnification: ×400.

The two main partners of DTH reactions are $CD4^+$ memory T lymphocytes and macrophages, which are both known targets for RANTES. This suggests that RANTES production plays a critical role in the accumulation of both cell populations in DTH granulomas, acting in concert with additional chemokines including MCP-1 or inducible protein [IP]-10 (16, 17). RANTES does not chemoattract naive T lymphocytes, B lymphocytes, or neutrophils (7), which may explain the paucity of such cells in DTH granulomas. Although it displays potent chemoattracting properties for eosinophils in vitro (18–20), eosinophils are absent in DTH reactions, suggesting that the in vivo effects of RANTES on its potential targets may be restricted by additional factors present in the microenvironment such as the relative production of Th1- vs. Th2-type cytokines.

RANTES was initially characterized as a product of activated T lymphocytes (9), and was subsequently reported to be also produced by kidney epithelial cells, platelets, and fibroblasts (6, 12, 18, 21). We show that macrophages and endothelial cells also produce RANTES. Macrophages are thus producers as well as targets of RANTES, suggesting that their recruitment in DTH granulomas may be self-perpetuating. Endothelial cells synthesize MCP-1, IL-8, and IP-10 (22) in addition to RANTES, and may thus have a dual effect on recruitment of immune cells. By expressing adhesion molecules, they may allow binding of circulating cells to their surface, and by expressing chemokines, they may trigger migration of these cells to the site of the immune reaction. The range of adhesion molecules and of chemokines

expressed by endothelial cells may play a pivotal role in selecting which cell populations will participate in the immune reaction. In DTH reactions, endothelial cells are not present in granulomas but in the inflammatory reaction surrounding them. From this site, they may selectively recruit circulating macrophages and $CD4^+$ memory T helper lymphocytes, which will then migrate to the granuloma.

Production of RANTES by macrophages and endothelial cells is itself integrated in a network of cytokine production. Synthesis of RANTES is upregulated by IFN-$\gamma$, IL-1$\beta$, and TNF-$\alpha$ (12, and this work). Interestingly, increased production of IFN-$\gamma$, IL-1$\beta$, and TNF-$\alpha$ is a specific feature of DTH granulomas (14, 23–28). This suggests that the self-perpetuation of DTH granulomas may result from a cascade of cytokine production as follows. Macrophages produce IL-1$\beta$ and TNF-$\alpha$ and $CD4^+$ memory Th1-type lymphocytes produce IFN-$\gamma$. These three cytokines act in synergy to induce production of RANTES, which leads to recruitment of additional macrophages and memory T helper cells in the granuloma. This action of IL-1$\beta$, TNF-$\alpha$, and IFN-$\gamma$ on RANTES production would complete their enhancing effect on adhesion molecule expression by endothelial cells (29–31). Such a self-renewing loop may shed light on the mechanism by which Th2 cytokines downregulate the formation of DTH granulomas. IL-4 and IL-10 each inhibit production of IL-1$\beta$, TNF-$\alpha$, and IFN-$\gamma$ (32–36), and IL-4 directly downregulates RANTES production (12).

This study provides clues to the selective accumulation of $CD4^+$ memory T lymphocytes and of macrophages inside DTH granulomas, an event that may be dependent on the in situ production of the RANTES chemokine. The involvement of macrophages and endothelial cells in this local production is also shown, thus widening the spectrum of RANTES-producing cells and outlining the role of both cell populations in the constitution of DTH granulomas.

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