

# Formation of Eosinophilic and Monocytic Intradermal Inflammatory Sites in the Dog by Injection of Human RANTES but not Human Monocyte Chemoattractant Protein 1, Human Macrophage Inflammatory Protein 1 $\alpha$ , or Human Interleukin 8

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## Summary

Equilibrium binding studies on canine mononuclear and granulocytic cells allow the identification of a single high affinity receptor for the human C-C chemokine RANTES (dissociation constant,  $14 \pm 8$  pM), that, in contrast to the human RANTES receptor, has no affinity for human macrophage inflammatory protein 1 $\alpha$  (hMIP-1 $\alpha$ ). A single intradermal injection of hRANTES in dog resulted in eosinophil- and macrophage-rich inflammatory sites within 4 h. Cell infiltration peaked at 16–24 h after hRANTES injection. There was histological evidence of intravascular activation of eosinophils at 4 h, although eosinophils in the vasculature and interstitium contained apparently intact granules. Monocytes were the predominant cells adherent to venular endothelium at 16–24 h. Human MIP-1 $\alpha$  elicited no response in canine dermis, whereas monocyte chemoattractant protein 1 caused mild perivascular cuffing with monocytes. In contrast, human interleukin 8 induced a neutrophilic dermal infiltrate that was maximal by 4 h after challenge. This provides the first direct evidence in vivo that RANTES has significant proinflammatory activity and, in addition, could be a mediator in atopic pathologies characterized by eosinophilic and monocytic inflammatory responses.

The eosinophil is recruited to inflammatory sites of early- and late-phase immediate hypersensitivity (1, 2), plays a significant role in the response to parasitic pathogens (3), is a major cellular component of the inflammatory response, and is implicated in mucosal damage in bronchial asthma (4). The mechanism(s) underlying eosinophil recruitment to inflammatory sites have yet to be completely defined. The first of these entails increased production and mobilization of eosinophils from bone marrow by a variety of factors including IL-3 and IL-5 (5, 6). Subsequently, there is intravascular priming of eosinophils followed by endothelial adhesion and transmigration mediated by the leukocyte-specific CD18 integrins and particularly by the very late antigen 4/vascular cell adhesion molecule 1 counter-receptor pair (7, 8), and stimulation of eosinophil chemotaxis and activation of secretion.

A broad range of chemically diverse chemotaxins have been described with shared activities on eosinophils and other leukocytes. C5a, platelet-activating factor, and leukotriene B<sub>4</sub>

(LTB<sub>4</sub>) are chemotactic for eosinophils and neutrophils, (1). RANTES, macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ), monocyte chemoattractant protein 1 (MCP-1), and IL-8 are members of the intercrine (9) or chemokine (10, 11) family of proinflammatory basic chemoattractant polypeptides. RANTES, MIP-1 $\alpha$ , and MCP-1 were defined initially as genes or proteins expressed in activated leukocytes or as small platelet-derived growth factor-inducible genes, and are structural members of the C-C branch of the chemokine family (11), based on the adjacent position of the first two of a highly conserved four-cysteine motif (9), whereas IL-8 was defined as a monocyte-derived neutrophil chemoattractant and is the paradigm of the C-X-C branch of the chemokines. Only RANTES and MIP-1 $\alpha$  are chemoattractants in vitro for eosinophils (12, 13), monocytes, and certain T lymphocyte subsets (14, 15). They have also been described to have variable effects on the activation of eosinophils and the stimulation of secretion in the presence of cytochalasin (13).

The contribution of C-C chemokines to inflammatory

recruitment in vivo has been obscured by conflicting data, and by a failure to characterize and correlate in vivo activity with receptor-ligand specificities and activities across species. Nevertheless, a variety of proinflammatory effects have been demonstrated by the injection of MCP-1 or MIP-1 $\alpha$  in vivo (16, 17). These include recruitment of either neutrophils or mononuclear cells, depending upon the specific cytokine. It has been suggested, without causal evidence, that these molecules play an important role in inflammatory recruitment leading to cell activation and directional migration of specific leukocyte subsets and contribute to activation of integrin-mediated adhesive events necessary for transendothelial migration (18). Accordingly, we sought to characterize the canine receptors for human C-C chemokines and directly evaluated human C-C chemokine activities in vivo by comparing the capability of human (h)RANTES, hMCP-1<sup>1</sup>, hMIP-17a, and hIL-8 to evoke dermal inflammation.

## Materials and Methods

**Cells.** Dog PBMC and granulocytes were prepared by density gradient centrifugation on 1.084 g/ml Percoll as described (19). THP-1 cells were cultured in IMDM with 10% fetal bovine serum (GIBCO BRL, Gaithersburg, MD).

**RANTES, MIP-1 $\alpha$ , MCP-1, and IL-8.** All recombinant human chemokines were purchased from Peprotech (Princeton, NJ) and had <0.1 ng LPS/ $\mu$ g protein as determined by the Limulus Amebocyte Lysis assay (Whittaker Bioproducts, Walkersville, MD). MIP-1 $\alpha$  was iodinated with Chloramine-T according to Siciliano et al. (20) and a sp act of 14  $\mu$ Ci/ $\mu$ g. Bolton-Hunter labeled RANTES and MCP-1 was purchased from New England Nuclear (Boston, MA) and each had a sp act of 260  $\mu$ Ci/ $\mu$ g.

**Binding Assays.** Binding of 10,000–20,000 cpm (50–100 pM) of <sup>125</sup>I-ligands in the presence of varying concentrations of unlabeled ligand to cells at room temperature was assayed in 50 mM Hepes/1 mM, CaCl<sub>2</sub>/5 mM, and MgCl<sub>2</sub>/0.5% BSA, pH 7.2. Competition assays were performed by premixing labeled and cold ligand and initiating the assay by the addition of cells. Activity retained on polyethyleneimine-treated filters (Glass fiber size C; Whatman Laboratory Products, Clifton, NJ) after washing in binding buffer with 0.5 M NaCl was counted in a gamma counter (LKB Instruments, Gaithersburg, MD). Binding constants were calculated and Scatchard analyses were performed on competition binding assays using the LIGAND program (Dr. P. J. Munson, National Institutes of Health, Bethesda, MD) and are shown as the mean  $\pm$  SD.

**Intradermal Chemokine Challenge.** Conscious 3-yr-old female beagles, parasite free at the time of challenge but having had some parasite exposure  $\sim$ 2 yr previously, were shaved and then subjected to skin challenge by intradermal injection at up to 12 skin sites. Doses of chemokine ranged from 10 to 500 pmol/site in a vehicle of PBS containing 0.1% human serum albumin, or with LPS in vehicle, or with vehicle alone as controls. At specific times (4–24 h) after intradermal injection of agonist, 6-mm punch biopsies of skin sites were obtained under anesthesia (thiamyl 2% to effect and 0.05 mg/kg acepromazine, i.m.), and the 5- $\mu$ m paraffin-embedded hematoxylin and eosin-stained histology was examined. Each dose of each chemokine was examined in duplicate sites in two dogs

<sup>1</sup> Abbreviations used in this paper: hMCP-1, human monocyte chemoattractant protein 1; hMIP-1 $\alpha$ , human macrophage inflammatory protein 1 $\alpha$ ; i.d., intradermal.

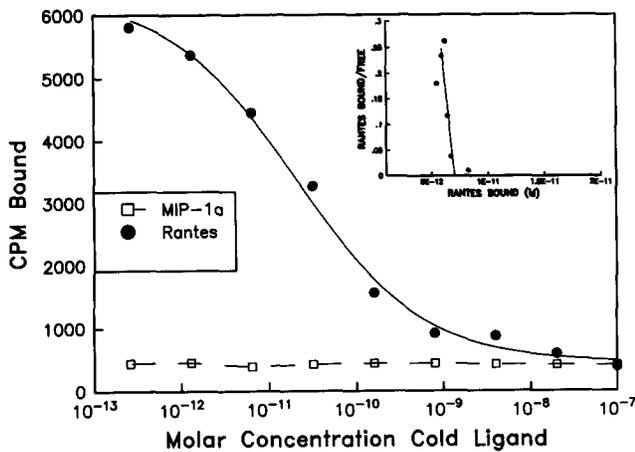
in four separate experiments (RANTES), three experiments for IL-8 and MCP-1, and a single experiment for MIP-1 $\alpha$ . Full blood counts and differentials were performed on EDTA-anticoagulated blood by Vet Research (Valley Stream, NY), and additional differential peripheral blood counts were performed on Diff-Quik (Baxter Healthcare, Glendale, CA) stained blood smears and are expressed as the mean  $\pm$  SD. New Zealand White rabbits (Hazelton Systems, Aberdeen, MD) and Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were skin challenged under ketamine-xylazine anesthesia and biopsied after euthanasia. *Escherichia coli* 0111:B4 LPS from Difco Laboratories (Detroit, MI) was used for injection. All animal experimentation was approved by and under the supervision of the Institutional Animal Care and Use Committee.

**Quantitation of Dermal Infiltrates.** Five coded serial 5  $\mu$ m hematoxylin and eosin-stained paraffin sections from each skin site were examined blind by at least two observers. Dermal infiltration was measured by counting the absolute numbers of extravascular eosinophils, macrophages, neutrophils, and lymphocytes at a magnification of 400 in a counting grid of 16 squares empirically determined to cover an area 24.5  $\times$  24.5  $\mu$ m. Dermal infiltrations were counted in each of three serial sections, from a minimum of four separate sites from four dogs, and expressed as the numbers of each cell type per mm<sup>2</sup>  $\pm$  SEM, rounded to the nearest ten. The numbers of separate skin site enumerated are shown (see Table 1). The typical low power appearance of sites quantified is shown (see Figs. 2 A, 3 C, 4 A, and 5 A). Dermal sites were specifically chosen because infiltration of the subdermal fat resulted in dense cords of leukocytes, too intense to permit adequate counting and differentiation of the leukocyte types by nuclear and cytoplasmic granular morphology.

## Results and Discussion

**The Canine RANTES Receptor Binds Human RANTES but not hMIP-1 $\alpha$ .** We previously demonstrated that dog mononuclear cells bound human RANTES and human MCP-1, and underwent a ligand-induced Ca<sup>2+</sup> flux when exposed to 100 nM RANTES or 10 nM MCP-1 (19). Equilibrium binding studies on the canine RANTES receptor on intact mononuclear cells showed a single receptor with  $K_d$  of 14  $\pm$  8 pM and 830  $\pm$  80 sites/cell (Fig. 1). Receptor assays were also performed on a mixed population of canine granulocytic cells, which displayed an identical binding constant but a lower site number of  $\sim$ 200 sites/cell. A similar site number of 190 sites/cell was obtained on a mixed population of human granulocytes, probably reflecting the small proportion of eosinophils present. The canine RANTES receptor has no measurable binding affinity for human <sup>125</sup>I-MIP-1 $\alpha$  (Fig. 1). In contrast, in our hands, and as reported by others (21–23), the RANTES/MIP-1 $\alpha$  receptor on human THP-1 membranes, binds both RANTES and MIP-1 $\alpha$ , which compete fully for each other, with  $K_d$  on isolated membranes of 70  $\pm$  20 and 200  $\pm$  20 pM, respectively (data not shown).

**Intradermal Injection of hRANTES in Dog Resulted in Eosinophilic and Monocytic Inflammatory Response.** Whereas human RANTES (500 pmol/site, intradermal [i.d.]) evoked no dermal response in rat and rabbit (data not shown) despite some specific binding to mononuclear cells (19), intradermal injection in the dog resulted in dose- and time-



**Figure 1.** Equilibrium binding cold displacement by RANTES of  $^{125}\text{I}$ -RANTES to canine PBMC (●). The points represent the means of triplicate measurements with a superimposed four-parameter fit. Scatchard analyses showed a single high affinity receptor for  $^{125}\text{I}$ -RANTES with  $K_d$  of  $14 \pm 7.8$  pM (inset).  $^{125}\text{I}$ -MIP-1 $\alpha$  failed to bind to canine PBMC (□). Data from one of two similar experiments are shown. Superimposable equilibrium competition binding was obtained on canine granulocytes.

dependent inflammatory responses in 15 of 16 sites analyzed. Cell counts of extravascular leukocytes derived from the tissue histology are summarized in Table 1. Fig. 2 shows the histology of RANTES- and vehicle alone-injected skin sites. Injection of as little as 10 pmol/site i.d. resulted in perivascular dermal cuffing with monocytes and eosinophils at 24 h (Fig. 2 B). Higher doses of 50 pmoles and 500 pmol/site resulted in inflammatory lesions of increasing severity, with diffuse, full-

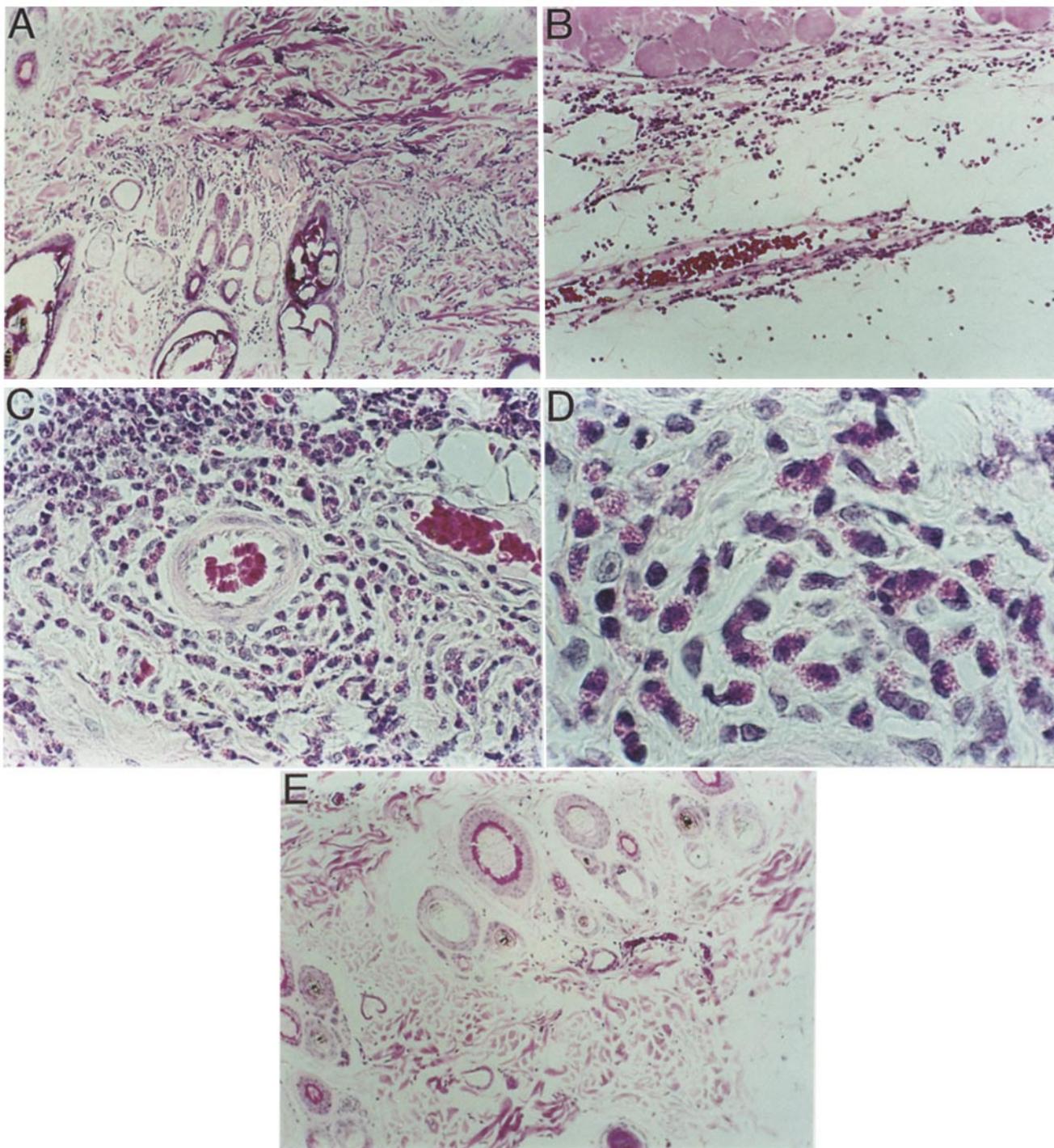
thickness dermal inflammation seen at the highest dose at 24 h (Fig. 2 A). At all dose levels of RANTES, the evoked responses were characterized by the subcutaneous accumulation of eosinophils, macrophages, and a smaller number of neutrophils on the periphery of lesions (Table 1, Fig. 3 C). The abundant subcutaneous perivascular eosinophilic infiltrate is shown in Fig. 2 C. The bulk of canine eosinophils recruited to sites of hRANTES injection have copious intact eosinophilic granules, and there is little histological evidence of RANTES-induced eosinophil degranulation in vivo (Fig. 2 D). Immunological evidence for extracellular eosinophil major basic protein could be a more sensitive measure of secretory responses. Dvorak (24) has documented degranulation of crystalline major basic protein-containing cores in vivo by electron microscopy without complete loss of granule contents. It should be noted that RANTES-injected skin sites showed no induration at 24 h, and no swelling was evident, though some separation of collagen fibrils could be seen histologically.

*The Kinetics of the Canine Response to Intradermal hRANTES Injection.* Within 4 h of hRANTES injection, eosinophilic and monocytic cuffing of dermal and subcutaneous venules was seen (Fig. 3 A). There was also evidence for intravascular eosinophil activation with adherence of eosinophils to endothelium and homotypic aggregation of eosinophils (Fig. 3 B), despite the presence of apparently intact cytoplasmic granules in extravascular eosinophils by light microscopy (Fig. 2 D). Intravascular leukocytes were not included in Table 1. Cell accumulation increased at 8 h and plateaued between 16 and 24 h (Fig. 2 A, Table 1). Few neutrophils were evident at the 4-h time point, whereas a significant peripheral neutrophilic infiltrate ( $12 \pm 1.4\%$  of recruited cells) was seen at the later time points, and was probably secondary to mono-

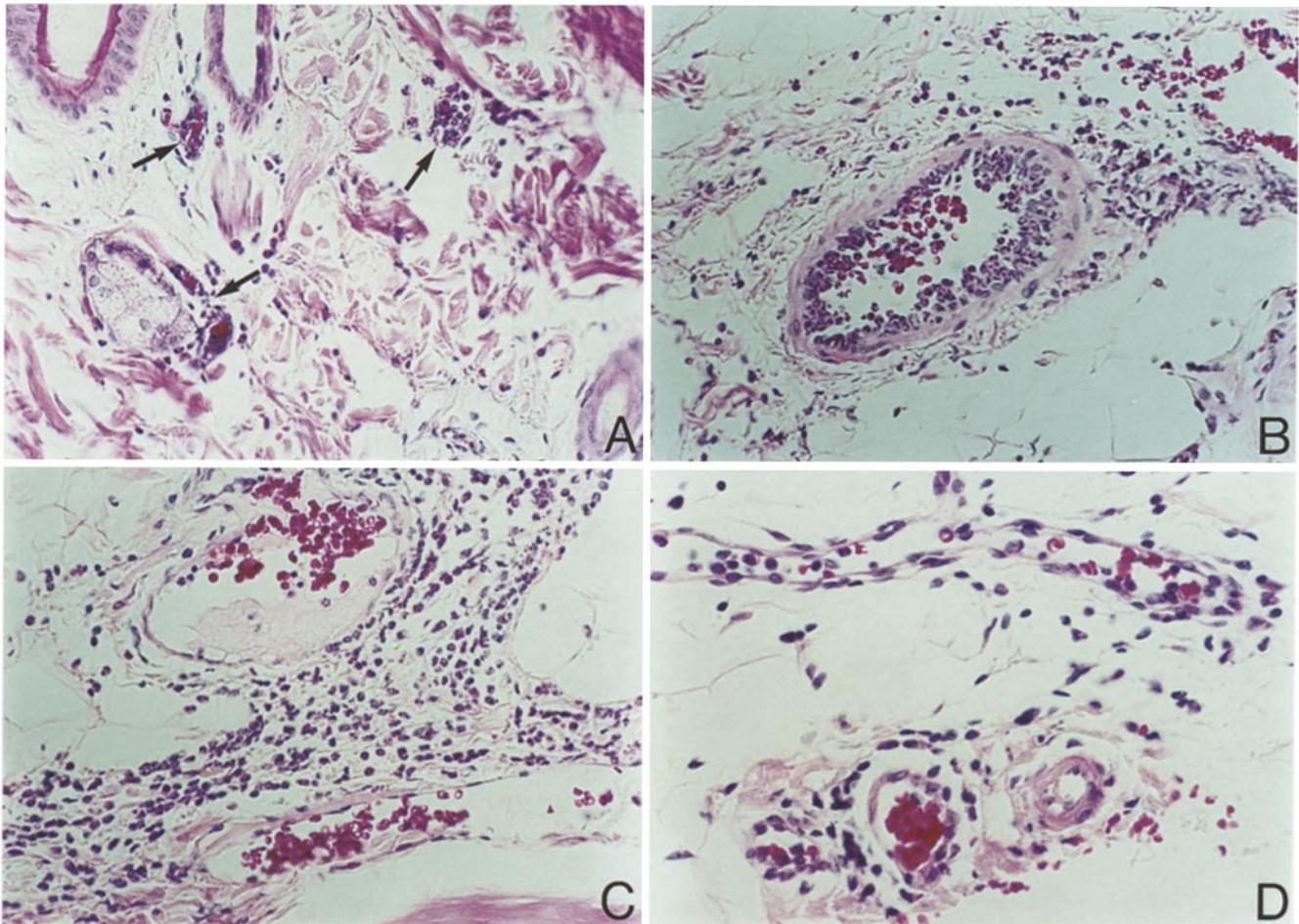
**Table 1.** The Numbers and Types of Leukocytes Infiltrating the Canine Dermis at Varying Times after Intradermal Injection of RANTES, MCP-1, IL-8, or Vehicle

Treatment	Time	Sites counted	PMN	Monocytes	Eosinophils	Lymphocytes
<i>pmol/site</i>	<i>h</i>					
Vehicle	24	8	0	180 ± 60	20 ± 10	0
RANTES (10)	24	4	80 ± 30	630 ± 100	350 ± 60	30 ± 10
RANTES (50)	24	4	270 ± 60	925 ± 110	780 ± 140	60 ± 20
RANTES (500)	4	4	70 ± 20	550 ± 40	340 ± 50	30 ± 10
RANTES (500)	8	4	190 ± 20	470 ± 30	420 ± 50	60 ± 10
RANTES (500)	16	4	330 ± 40	900 ± 230	750 ± 100	70 ± 20
RANTES (500)	24	8	350 ± 40	1,200 ± 110	1,480 ± 160	140 ± 20
IL-8 (500)	4	4	2,740 ± 280	100 ± 20	90 ± 20	40 ± 10
IL-8 (500)	24	4	1,810 ± 180	170 ± 40	120 ± 30	390 ± 70
MCP-1 (500)	24	4	30 ± 10	610 ± 220	20 ± 10	60 ± 10
MIP-1 $\alpha$ (500)	24	4	120 ± 20	320 ± 70	70 ± 40	0

Results reflect cell counts from three serial sections from the minimum of four separate skin sites from four animals. Cell counts (rounded to the nearest 10) are expressed as the mean ± SEM of cells/mm<sup>2</sup>.



**Figure 2.** Hematoxylin- and eosin-stained sections of 24-h dermal responses to RANTES. (A) High dose (500 pmol/site) RANTES challenge resulted in full-thickness dermal inflammation.  $\times 100$ . (B) Low dose (10 pmol/site) RANTES injection resulted in diffuse perivascular cuffing by monocytes and eosinophils.  $\times 160$ . (C) The predominant lesions were eosinophil- and macrophage-rich perivascular infiltrates, with abundant intact eosinophils.  $\times 400$ . (D) Little histological evidence of release of eosinophil granules into the interstitium can be seen at high power, which show intact eosinophils with characteristically large cytoplasmic granules.  $\times 1,000$ . (E) Vehicle-injected control site.  $\times 100$ .



**Figure 3.** Hematoxylin- and eosin-stained sections showing the kinetics of the dermal response to exogenous human RANTES. (A) 4-h RANTES sites show eosinophilic and monocytic cuffing of small venules (arrows)  $\times 250$ . (B) Evidence for intravascular activation of eosinophils by RANTES at 4 h. Vessels show adherent eosinophils and intravascular homotypic eosinophil aggregates adherent to the vessel wall.  $\times 250$ . (C) Some neutrophil infiltration is seen in addition to eosinophils and monocytes by 16 h.  $\times 250$ . (D) At 24 h, monocytes are the major cell type adherent to small vessel endothelium.  $\times 400$ .

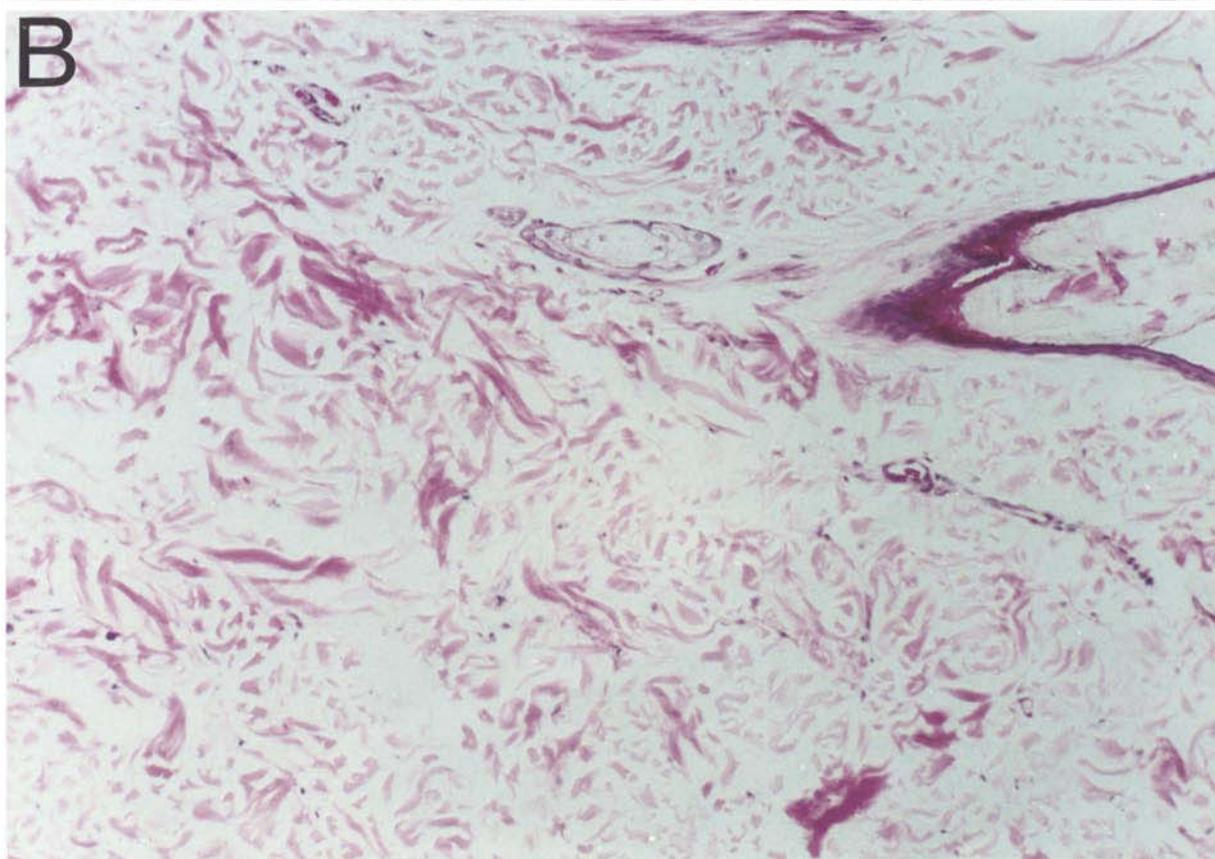
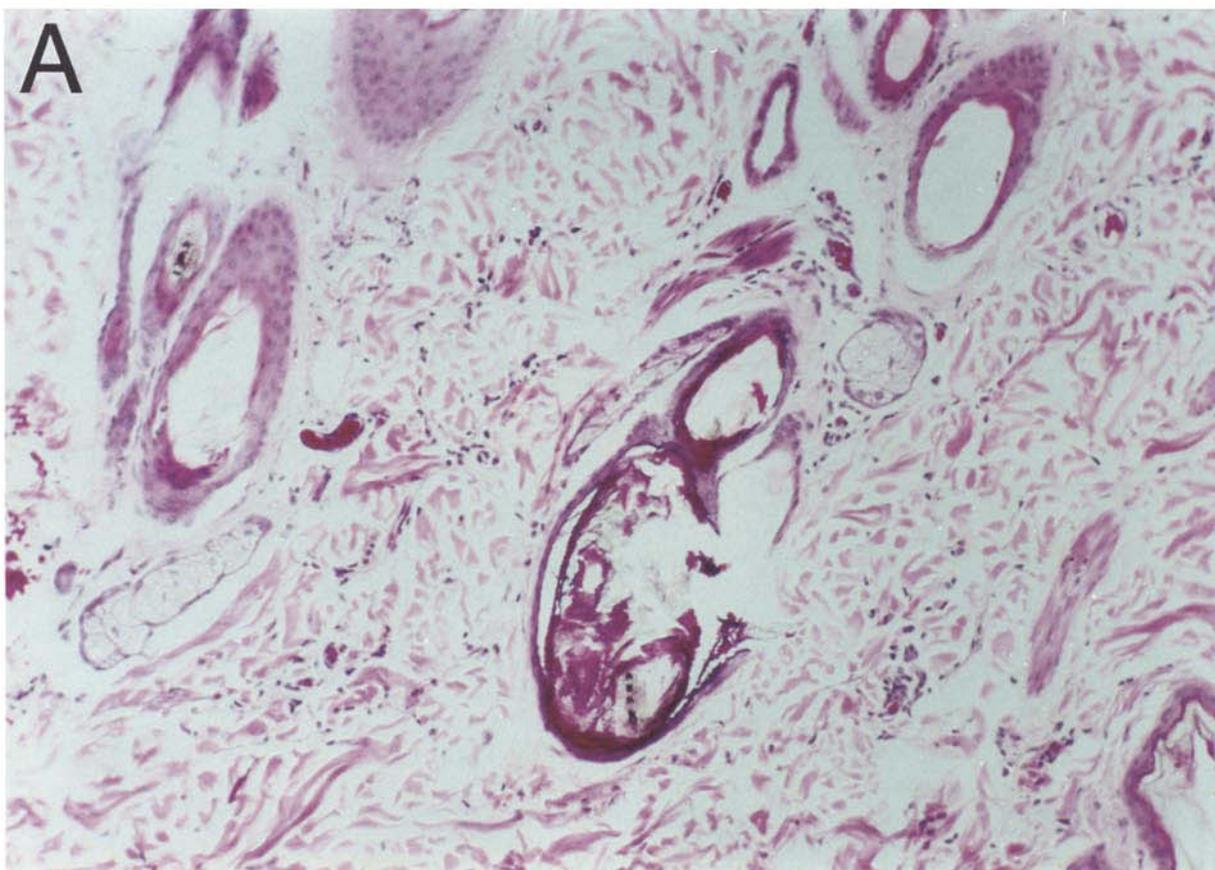
cyte and eosinophil recruitment (Fig. 3 C, Table 1). At 4 h, the eosinophil was the predominant cell adherent to the post-capillary venular endothelium, whereas the endothelial adherent leukocytes at 24 h were mainly monocytic (Fig. 3 D). The adherent of cells to endothelium visible in tissue sections is but a static moment in the dynamic formation and maintenance of an extravascular inflammatory site, and may not correlate exactly with the numbers and types of leukocytes quantified in Table 1.

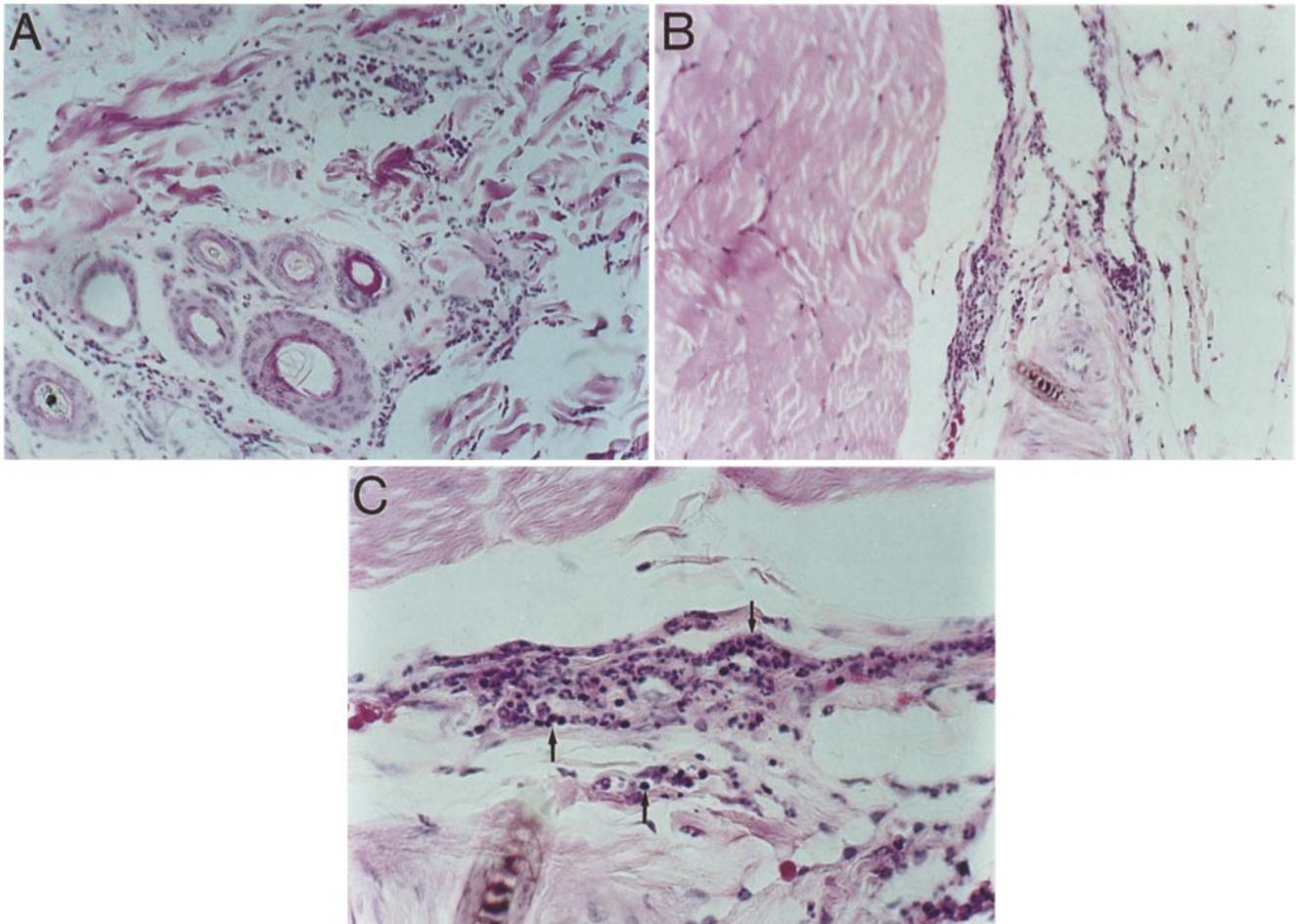
*Comparison of the RANTES Response with Equivalent Doses of hMCP-1.* To contrast potential differences between the monocytic and eosinophilic response to RANTES, we simultaneously compared its *in vivo* effects to those of the monocyte-specific C-C chemokine hMCP-1. hMCP-1 injection produced mild perivascular cuffing and dermal infiltration by monocytes at 500 pmol/site (Fig. 4 A, Table 1). This was at a dose  $\sim 50$ -fold-greater than that needed to elicit a reproducible response to RANTES. We have now examined MCP-1 responses to human chemokines in rat, guinea pig, rabbit, dog, and

rhesus. In all cases, we only see leukocyte recruitment when there is both high affinity binding (19) and a ligand-dependent  $Ca^{2+}$  flux (i.e., in dog and rhesus). Rat cells did not bind MCP-1 and rabbit cells did not undergo a ligand-dependent  $Ca^{2+}$  flux (19). Despite published reports to the contrary (16, 17) in rat and rabbit, we saw no significant responses to MCP-1 in those species. A similar inability to reproduce the results upon injection of hMCP-1 into rat and rabbit have been alluded to by Yoshimura and Yuhki (25). The results obtained in canines are similar to those shown by Yoshimura in autologous guinea pig MCP-1 cutaneous challenges (26). hMCP-1 alone in the canine is a less effective proinflammatory stimulus on a molar basis than RANTES or IL-8.

As expected from the failure to demonstrate specific binding of hMIP-1 $\alpha$  to canine mononuclear cells, negligible dermal responses were elicited by up to 500 pmoles/site of this chemokine.

*The Dermal Response to hIL-8 Peaks by 4 h and Persists at 24 h.* hIL-8 evokes an abundant neutrophilic infiltrate that





**Figure 5.** Hematoxylin- and eosin-stained sections of hIL-8-injected skin sites. (A) hIL-8 induced a pronounced neutrophilic infiltrate at 4 h.  $\times 250$ . (B) Some PMN were still detectable at 24 h, though the area of infiltrate was somewhat diminished.  $\times 160$ . (C) A subpopulation of small lymphocytic cells could also be seen at 24 h (arrows).  $\times 400$ .

was maximal within 4 h after injection and still persists at 24 h (Fig. 5, A and B, and Table 1). By 24 h, a distinct subpopulation of small lymphocytic cells in addition to the predominant neutrophils, can be seen at hIL-8 sites (Fig. 5 C). The early peak of hIL-8 response (4 h) compared to RANTES (16 h–24 h) could perhaps reflect the kinetics and life spans of the responding leukocyte subsets, and their relative abundance in the peripheral blood. In these experimental animals, neutrophils are the most abundant leukocyte, and also migrate the fastest *in vitro*. Additional possibilities are the different rates of *in vivo* metabolism of the two chemokine families, for which no data currently are available. It has been recently reported (27) that IL-8 is inactivated by the C5a-inactivating protease. The difference in kinetics of migration probably does not reflect any physical difference between the C-X-C and C-C chemokines such as binding to negatively charged extracellular matrix, as they are all highly conserved basic pro-

teins, with theoretically superimposable structures (28). A third explanation could be that RANTES evokes a series of indirect effects leading to longer-term recruitment of eosinophils and monocytes, and Ab neutralization studies would allow indirect effects to be quantified.

*The Proinflammatory Activity of RANTES Is Not LPS Mediated.* The proinflammatory activity of hRANTES cannot be explained by LPS contamination. LPS levels were  $<100$  pg/ $\mu$ g of protein and these protein preparations were inactive in the same dose range in rabbit and rat, both LPS-responsive species. Also, injection of 0.4 ng of LPS in vehicle as a control during these experiments resulted in a very mild perivascular monocytic infiltrate at 24 h, with some neutrophils adherent to the endothelium of small venules, a histological response very different from that seen with hRANTES.

The property of hRANTES to recruit eosinophils and monocytes to extravascular sites is distinct, and different from

**Figure 4.** Hematoxylin- and eosin-stained sections of the canine dermal response to exogenous hMCP-1. (A) hMCP-1 at 500 pmol/site induces a mild perivascular monocytic infiltrate. In contrast to the responses to hRANTES and hIL-8, no evidence of subcutaneous cellular infiltrates could be seen with hMCP-1. (B) Vehicle-injected control skin site. A and B,  $\times 100$ .

**Table 2.** *Peripheral Blood Leukocyte Counts from the Experimental Beagles*

White blood cells	10.4 ± 1.6 × 10 <sup>6</sup> /ml
Neutrophils	52.0 ± 3.6%
Lymphocytes	28.0 ± 5.2%
Monocytes	10.7 ± 1.5%
Eosinophils	9.3 ± 2.3%

Basal white cell counts were obtained from four animals. Results are expressed as the mean ± SD for the group of dogs.

the responses elicited by hIL-8 and the human C-C chemokine MCP-1, both of which were injected simultaneously at separate sites as controls in each experiment. The cellular composition of each inflammatory site reflected the chemokine injected at the local site, suggesting that chemokines are locally active. These data are not in keeping with the possibility that chemokines are acting systemically under these conditions.

Canines have a normal range of peripheral blood eosinophils of between 2 and 10%. These dogs were near the upper limit of the normal range, were helminth free but had some prior exposure to parasites. The relative abundance of peripheral blood eosinophils (Table 2) and perhaps eosinophil priming may have contributed to the florid nature of the RANTES response in these animals. This possibility could best be ana-

lyzed in a small animal system, where reproducible priming could be experimentally obtained, but would require using either mouse or guinea pig RANTES. In those systems, the extent of eosinophil recruitment could perhaps be most quantitatively assessed by the use of a biochemical marker for the presence of eosinophils such as major basic protein.

These data suggest that RANTES could play a significant role in diseases characterized by eosinophilia and the mobilization of eosinophils and monocytes to sites of tissue damage such as the asthmatic lung (2) and atopic dermatitis (29). This report provides the first direct evidence in vivo that the selective migration of leukocyte subsets to C-C chemokines such as RANTES in vitro, has some correlation in vivo, and supports the caveat that studies in vivo with exogenous cytokine across a species barrier can be adequately interpreted only when performed in concert with detailed characterization of the target receptor and the functional sequelae of receptor occupancy and activation by the exogenous cytokine.

A critical problem facing the chemokine field has been the need to demonstrate a relationship in vivo between chemokine presence, the presence of specific, activatable receptors, and leukocyte recruitment. This has now been achieved at least for RANTES and IL-8 (30). Additional approaches including neutralizing mAbs or chemokine gene deletion by homologous recombination, together with a detailed understanding of these ligands and their receptors, should then facilitate our understanding of the factors regulating leukocyte recruitment to sites of tissue damage and repair.

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We thank Dr. Gerard Hickey for insights into cutaneous responses in canines and Reid Ziegler and Ron Matura for Photomicroscopy.

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*Received for publication 10 June 1993 and in revised form 3 September 1993.*

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