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Expression and Secretion of RANTES (CCL5) in Granulomatous Calcified Tissue before and after Lipopolysaccharide Treatment *In Vivo*

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Abstract. RANTES (regulated on activation, normal T cell-expressed and secreted) is a CC chemokine appearing to be involved in the recruitment of leukocytes at inflammation sites. RANTES is produced by $CD8^+$ T cells, epithelial cells, fibroblasts, and platelets. It acts *in vitro* in leukocyte activation and human immunodeficiency virus suppression, but its role *in vivo* is still uncertain. In our study, we established the involvement of RANTES in an *in vivo* model of chronic inflammation induced by potassium permanganate, leading to calcified granulomas. In our rat model, RANTES expression (mRNA and protein) was significantly upregulated in granulomatous tissue; RANTES expression was further increased upon i.p. injection of lipopolysaccharide (LPS), while it was kept at basal levels by dexamethasone (Dex) given 18 hours before sacrifice. LPS and Dex increased and decreased, respectively, the recruitment of mononuclear cells in granulomatous tissue compared with control granulomas from phosphate-buffered saline (PBS)-treated animals. In granuloma tissue, levels of RANTES were higher in LPS-treated rats and lower in the Dex group compared to controls. RANTES was also found in the conditioned medium of granuloma tissue from treated (LPS or Dex) and untreated (PBS) rats. When LPS was added *in vitro* for 18 hours, RANTES was further increased, except in the Dex group ($P > 0.05$). On serum analysis, RANTES levels were higher in the LPS group and lower in the Dex group compared to controls. This study shows for the first time that RANTES is produced *in vivo* in chronic, experimental inflammatory states, an effect increased by LPS and inhibited by Dex.

Key words: Calcified granuloma — RANTES — CCL5 — Lipopolysaccharide — Inflammation — *In vivo*

Chemokines are chemotactic cytokines principally involved in the mobilization of cells of the immune system in various physiological and pathological states [1–7]. Depending on the position and the number of conserved cysteine residues within the primary amino acid sequence, the chemokines are conventionally divided into four subfamilies: CXC (alpha), CC (beta), C (gamma), and CX3C (delta). Among the CC chemokine subfamily, one member is RANTES (regulated on activation, normal T cell-expressed and secreted), also called CCL5, which has strong proinflammatory properties [8–12]. RANTES can deliver a signal upon binding to multiple receptors on the cell surface in the seven-transmembrane G protein-coupled receptor family, namely CCR1, CCR3, CCR4, CCR5, and the cytomegalovirus receptor US28. RANTES is secreted by various cell types including platelets, mesangial cells, fibroblasts, macrophages, endothelial cells, some epithelial cells, antigen-specific $CD4^+$ T-helper cells, and effector $CD8^+$ T cells. Similar to other CC chemokines, RANTES is a chemoattractant for monocytes, unstimulated memory T cells ($CD4^+/CD45RO^+$), stimulated $CD4^+$ and $CD8^+$ T cells, basophils, eosinophils, and mast cells but not neutrophils. RANTES plays a major role in the magnitude and polarity of the T-cell response [13–16]. Moreover, it has been found to be an inhibitor of human immunodeficiency virus (HIV) replication *in vitro* and to possess a suppressive effect on HIV, probably by binding on $CD4^+$ T cells the CCR5, the major coreceptor for cell entry of the virus [17–19]. RANTES is an activator for basophils and mast cells [20–23], where it induces the production and release of histamine, and for eosinophils [24–27], causing the release of specific inflammatory mediators and upregulating the adhesion molecules CD11b and CD18.

Besides the induction of leukocyte migration, this chemokine shows the peculiar capabilities of self-aggregating on the cell surface of leukocytes and strongly activating them *in vitro* [28]. Therefore, the biological effects of RANTES seem to be dependent on

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its concentration and degree of multimerization. However, this is still to be demonstrated *in vivo*.

In our study, we induced an experimental chronic inflammatory state in rats by subcutaneous injection (0.2 mL) of a saturated water solution (1:40) of potassium permanganate ($KMnO_4$). This treatment causes the formation of calcified granulomatous tissue at the site of injection, reaching an apex in size and weight after 1 week. We found that RANTES mRNA is upregulated in granulomatous tissues compared to controls (treated with phosphate-buffered saline [PBS]). The intraperitoneal (i.p.) administration of lipopolysaccharide (LPS, 6 μg/200 μL bolus injection per rat) 18 hours before sacrifice, which promotes granuloma formation [29], produced an increase of RANTES mRNA in granulomatous tissue compared to controls. On the contrary, i.p administration of the anti-inflammatory drug dexamethasone (Dex, 300 μg/200 μL bolus injection per rat) [30], which inhibits the formation of calcified tissue, decreased RANTES mRNA. In conditioned medium from homogenized tissue and serum, levels of soluble RANTES were higher ($P < 0.05$) in LPS-treated animals and lower ($P < 0.05$) in the Dex group compared to controls (PBS). Similar results were obtained in the serum and in minced granulomatous tissue, where samples were further incubated *in vitro* with LPS (100 ng/mL) overnight. A strong increase ($P < 0.01$) in RANTES in all samples was detected but not in the minced granulomatous tissue from Dex-treated animals.

Materials and Methods

Animals and Experimental Design

Wistar rats (Stefano Morini, S. Polo d'Enza, Italy) were used, 250–280 g in weight, approximately 3 months of age, and raised under the same environmental and feeding conditions. Granuloma formation was experimentally induced according to a procedure previously described [31]. In brief, all rats received four dorsal subcutaneous injections (0.2 mL) of a saturated water solution of potassium permanganate diluted 1:40. At the sixth day, rats were divided into three groups of 12 animals each: one group receiving PBS (200 μL) i.p., one group treated with LPS from *Escherichia coli* (serotype 0111:B4) at 6 μg/200 μL bolus injection per rat, and the other treated with Dex 300 μg/200 μL bolus injection per rat. Eighteen hours after treatments (7 days after $KMnO_4$ injections), rats were anesthetized and killed. Granulomas that formed at the injection sites in animals from all experimental groups were measured, and the mean of the major diameter was calculated for each group and expressed in millimeters. Also, the average weight, expressed in grams, of freshly enucleated granulomas was calculated for each group. Some of the granuloma biopsies were prepared for histochemistry and analyzed under the optic microscope. Some of the fresh granulomas were immediately subjected to mRNA extraction and cDNA synthesis; others were rapidly homogenized in ice-cold PBS and tested for the presence of RANTES protein by enzyme-linked immunosorbent assay (ELISA). In another set of experiments, granuloma tissues from untreated animals (PBS) were enucleated, finely minced, vortexed in RPMI-1640, and incubated in the same medium plus 5% fetal calf serum in a test tube for 18 hours, while parallel samples were treated *in vitro* with LPS (100 ng/mL). Aliquots from supernatants were then tested by ELISA for RANTES release.

Experimental procedures involving animals were performed in compliance with the Guiding Principles in the Care and Use of Animals, approved by the Council of the American Physiological Society (http://www.the-aps.org/publications/journals/guide.htm).

Histological Analysis of Granuloma Tissue

After granuloma induction by $KMnO_4$, rats were i.p.-treated with PBS, LPS, or Dex 18 hours before sacrifice. The animals were divided into groups of three, each group having three controls. Granulomatous tissues were enucleated and immersed in a fixative solution. Slides were prepared with sections of tissue, stained with Giemsa/May-Grünwald, and analyzed under the optic microscope.

Morphological Studies (Immunofluorescence)

For immunofluorescence, a portion of granulomatous tissue was snap-frozen in isopentane previously chilled in liquid nitrogen. Cryostat sections (4 μm), after air drying and fixation in acetone for 1 minute, were washed in PBS and incubated for 30 minutes with 1:10 dilutions of fluoresceinated antibody specific for mouse immunoglobulin G (IgG) or RANTES. Finally, sections were washed for 10 minutes in PBS, mounted with 90% glycerol in PBS, coded, and randomized before examination. Sections were examined interdependently under an optic fluorescence microscope (×10, ×20 ×40; Diaphore THD; Nikon, Tokyo, Japan) by two observers.

von Kossa Staining on Calcified Granuloma

Calcium usually precipitates *in vivo* and *in vitro* tissue conditions. Calcification of the granuloma was assessed using von Kossa stain. The tissue was washed with PBS, and cultures were treated with 5% silver nitrate solution in the dark at 37°C for 30 minutes. The excess silver nitrate solution was then completely washed away using double-distilled H_2O, and the culture plate was exposed to sodium carbonate/formaldehyde solution for a few minutes to develop color. Calcified tissue could be distinguished by its von Kossa-positive staining (red-dark brown area). To evaluate calcified tissue, von Kossa-stained areas were viewed by light microscopy. Each experiment was performed in triplicate, and a minimum of three samples were analyzed each time.

Granuloma Tissue Processing for RANTES Determinations

Rat granuloma and control tissues were placed in ice-cold PBS, centrifuged at 200 × *g* for 5 minutes, and homogenized in 2 mL radioimmunoprecipitation assay buffer (1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 100 μg/mL phenylmethyl sulfonyl fluoride, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 5 mM ethylenediaminetetraacetic acid [EDTA] in PBS) with a Polytron (Brinkmann Instruments, Westbury, NY) for 30 seconds. During these procedures, tissues were kept on ice. The homogenate was centrifuged at 15,000 × *g* at 4°C for 10 minutes, and the supernatants were analyzed by ELISA for RANTES production. Protein concentration was assayed using bicinchoninic acid reagents (Sigma, St. Louis, MO).

In another set of experiments, granuloma tissues were finely minced and a precise amount was placed in RPMI-1640 in plastic test tubes. Each granuloma from LPS (6 μg/200 μL bolus injection per rat) or Dex (300 μg/200 μl bolus injection per rat) or PBS treatment as described above was exposed only

to the vehicle or exogenous LPS (100 ng/mL) for overnight (16–18 hours) incubation. Afterward, test tubes were centrifuged at 3,000 rpm at 4°C for 10 minutes and the levels of RANTES in culture supernatants were determined by a specific ELISA.

Serum Collection

Peripheral blood was obtained from intracardiac puncture of anesthetized rats, from 7-day $KMnO_4$ treatments, and collected in polypropylene tubes. Animals were treated with LPS or Dex for 18 hours before sacrifice. Serum was collected by whole-blood centrifugation (2,000 rpm for 10 minutes) after clot formation. The serum was then collected and tested for RANTES generation by ELISA.

ELISA of RANTES

The contents of RANTES in granuloma homogenates (containing equal amounts of total protein), granuloma-conditioned medium, and serum were determined using the specific sandwich ELISA for rat RANTES Duoset cytokine kit (R&D Systems, Minneapolis, MN), according to the manufacturer's recommendations. Recombinant rat RANTES (Peprotech, London, UK) was used as a standard. The results were normalized by total protein content in granuloma homogenates or by cell numbers.

RT-PCR Assay of RANTES Chemokine mRNA

Total RNA was extracted using TRIzol reagent (GIBCO BRL, Milan, Italy). Total RNA (5 μg) was reverse-transcribed (RT) according to the manufacturer's protocol (Superscript II Preamplification System, GIBCO BRL) using oligo(dT) as primer. cDNA prepared from 0.5 μg RNA was subjected to polymerase chain reaction (PCR) using the following rat RANTES specific primers (Invitrogen, Milan, Italy): forward 5′-catccctcaccgtcatc-3′, backward 5′-gctcatctccaaatagttg-3′ (expected amplimer size 257 nt). The primer pair spanned at least one intron in the corresponding genomic DNA. Positive RNA controls and normal muscle tissue were used to confirm the specificity of primer pairs (data not shown). Negative controls were performed by omitting the RT step or the cDNA template from PCR amplification. The target sequence was amplified at 56°C between 22 and 32 cycles in order to yield visible products within the linear amplification range. Controls were PCR products separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide. The RT-PCR band at the expected size was also directly sequenced to confirm its identity. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward 5′-gcaagttcaacggcacagtcaaggctg-3′, backward 5′-gcccaggatgccctttagtgggccctc-3′; expected amplimer size 670 nt) was used as internal control. Signals were acquired and quantified by the Gel Doc EQ System using Quantity One software (Bio-Rad, Milan, Italy).

RANTES mRNA Detection by Northern Blot

RNA (10 μg/lane) was electrophoresed through a 1% agarose, 2.2 M formaldehyde denaturing gel with Maintenance Operation Protocol (MOP) buffer, followed by capillary transfer to nylon membranes. After transfer, RNA integrity was assessed by methylene blue staining (not shown). After baking for 2 hours at 80°C, the filters were prehybridized at 65°C in 0.5 M $NaHPO_4$ buffer (pH 7.0), containing 1 mM EDTA, 7% SDS, and 1% bovine serum albumin. Then, filters were hybridized with ^{32}P-labeled cDNA probes and 100 μg/mL salmon sperm DNA at 65°C overnight. cDNA probes were labeled with ^{32}P by the random priming method (Megaprime DNA labeling system; Amersham, Milan, Italy). The filters were exposed to film and autoradiographed at −80°C after being washed. Band intensities on the autoradiogram were quantitated by laser scanning densitometer (GS-670, Bio-Rad). The filters were also reprobed with GADPH cDNA to correct for variation in RNA loading and transfer efficiency. cDNA probes were made by RT-PCR. As a template, total RNA extracted from LPS-stimulated rat peritoneal macrophages was used for first-strand cDNA synthesis. After RT, PCR was performed in a total volume of 50 μL with initial denaturation at 94°C for 3.5 minutes, followed by 30 cycles with the following sequential steps: denaturation at 94°C for 1.5 minutes, annealing at 54°C for 1.5 minutes, and elongation at 72°C for 1.5 minutes. Samples were incubated at 7°C for 10 minutes before completion. PCR primers were synthesized (Invitrogen) based on the published cDNA sequences for rat RANTES (GenBank): forward 5′-gatctctgcagctgcatcc-3′, backward 5′-cttctctgggttggcacac-3′; GADPH forward 5′-tcaccatcttccaggagcg-3′, backward 5′-ctgcttcaccaccttcttga-3′. PCR products were electrophoresed on an ethidium bromide-stained 1% agarose gel. Gene-specific bands were visualized under ultraviolet light. RT-PCR fragment sequences (RANTES 232 nt, GADPH 572 nt) were 100% identical to those of the corresponding rat cDNA sequences from the GenBank database (data not shown).

Statistical Analyses

Data from different experiments were combined and reported as the mean ± standard deviation (SD). Student's *t*-test for independent means was used for statistical analyses, with $P < 0.05$ being considered significant.

Results

Subcutaneous Induction of Granuloma Formation

In this study, we used experimental granuloma formation induced by $KMnO_4$ as a good and valid model of chronic inflammation and granuloma calcium accumulation [32–34]. Fig. 1A shows the subcutaneous injection sites (arrows) of $KMnO_4$ saturated solution, diluted 1:40. Fig. 1B shows an example of granuloma formation after 7 days' induction. The presence of calcium in the granuloma was demonstrated by incineration of the tissue at 180°C for 4 hours (data not shown).

Histological Examination of Granuloma Tissue

We studied the involvement of the CC chemokine RANTES in the formation of experimental granuloma *in vivo*. For this purpose, we used a validated model for chronic inflammation, developed in our laboratory, which is based on subcutaneous dorsal administration of $KMnO_4$ in experimental rats. The rats received a dorsal subcutaneous injection (0.2 mL) of a saturated water solution (1:40) of $KMnO_4$, a strong oxidant compound that, at high concentration, can induce inflammatory reactions and granuloma formation at the site of injection.

In Fig. 2, a representative microscopic view (×20) of granuloma tissue sections is shown from (A) untreated (PBS) and (B) LPS-treated animals. Tissues were stained with Giemsa-May-Grünwald, and mononuclear cells

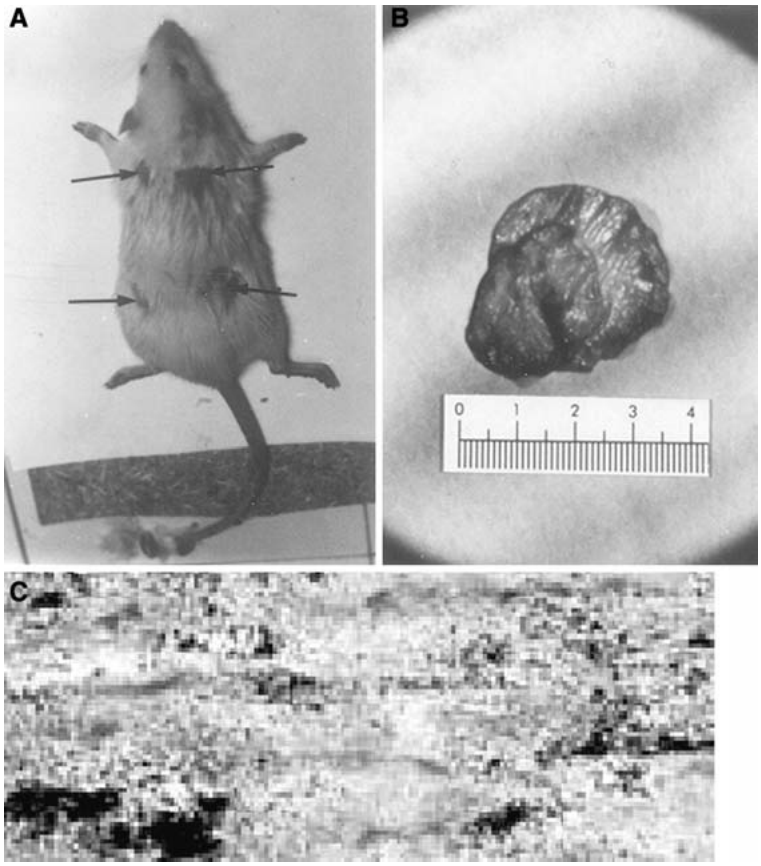

Fig. 1. (**A**) In this figure, the *arrows* indicate injection sites. Mice received four dorsal subcutaneous injections of 0.2 mL each of a saturated solution (diluted 1:40) of KMnO4 crystals. (**B**) An enucleated fresh granuloma tissue formed 7 days after subcutaneous injection of KMnO4. (**C**) Photomicrograph (×10 magnification) of granuloma tissue induced by $KMnO_4$ from untreated rat. The granuloma was assessed using von Kossa stain (brown area).

were counted under an optic field, using a grating size of 5×5 mm^2. Fig. 2B (rat treated with LPS 6 μg/200 μL bolus injection) shows a marked increase ($P < 0.05$) in the number of mononuclear cells compared with control (PBS-treated, Fig. 2A).

Immunofluorescence Microscopy of Granuloma Tissue

Stained tissues were examined with fluorescence microscopy to locate the IgG antibody. By immunofluorescence microscopy, untreated rats (saline group) exhibited a decreased number of inflammatory cells (Fig. 3A) compared to LPS-injected animals (Fig. 3B). Rats treated with LPS showed an increase of IgG deposition, higher than the saline-treated rats (approx. 40%). Cells were activated for RANTES production as reported (see Figs. 4 and 5).

RANTES mRNA Expression in Calcified Granuloma Tissue

In order to determine RANTES mRNA expression in the induced granuloma tissue from LPS, Dex, or PBS (control) i.p.-treated animals, semiquantitative RT-PCR and Northern blot analyses were performed. In Fig. 4, an example of agarose gel electrophoresis of RT-PCR products is shown: in rats treated with LPS (6 μg/200 μL bolus injection), there was an increase of RANTES cDNA compared with the negative control (PBS-treated), whereas Dex (300 μg/200 μL bolus injection) produced the opposite effect. Similarly, RT-PCR results were confirmed in Northern blot experiments shown in Fig. 5: steady-state levels of RANTES mRNA in granuloma calcified tissue were generated, which significantly increased when rats were treated with LPS compared to PBS or Dex. In both types of experiment, LPS-activated rat peritoneal macrophages were used as a source of RANTES mRNA and used as positive control [35].

Production of RANTES from Homogenized Calcified Granuloma Tissue

Table 1 shows the concentration of RANTES (pg/mL/100 mg tissue) released in the supernatants of homogenized granuloma tissue from animals treated or not (control) with LPS or Dex 18 hours before sacrifice. The values represent two sets of four experiments in triplicate. LPS treatment strongly augmented ($P < 0.05$) the production of RANTES in the supernatant of homogenized granuloma tissue, whereas it was significantly inhibited by Dex compared to controls (PBS).

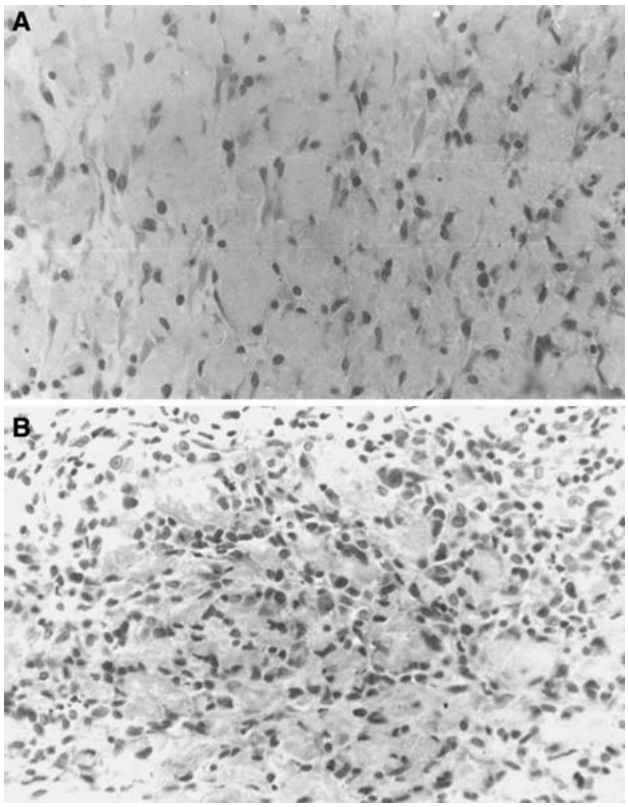

Fig. 2. Optic microscope view (×20) of mononuclear cells from a biopsy of enucleated granuloma tissue formed from $KMnO_4$ treatment after 7 days (0.2 mL/bolus injection/site). (**A**) Control granuloma tissue from PBS (200 μL)-treated rat. (**B**) Granuloma tissue from LPS (6 μg/200 μL) i.p.-treated rat. A significant increase ($P < 0.05$) of mononuclear cells is present compared to the PBS-treated control.

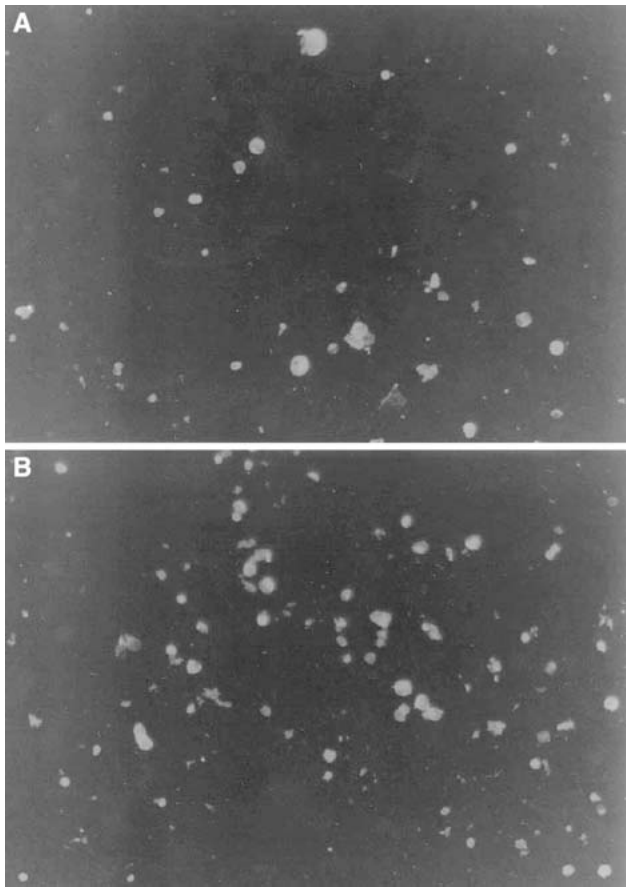

Fig. 3. Immunofluorescence micrographs. Representative sections of granuloma tissue from untreated rats (A, PBS 200 μL bolus injection/rat) and LPS-treated rats (B, 6 μg/200 μL bolus injection/rat). Sections were examined under an optic fluorescence microscope at ×20 magnification.

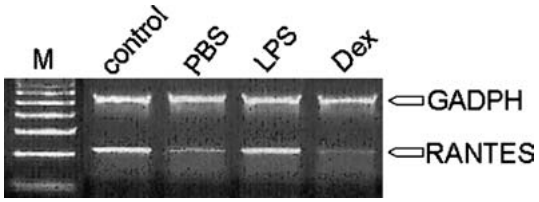

Fig. 4. RT-PCR for RANTES mRNA from granuloma tissue i.p.-treated with PBS (negative control), Dex, or LPS. Animals were i.p.-treated 18 hours before sacrifice. LPS-activated rat peritoneal macrophages were used as positive control.

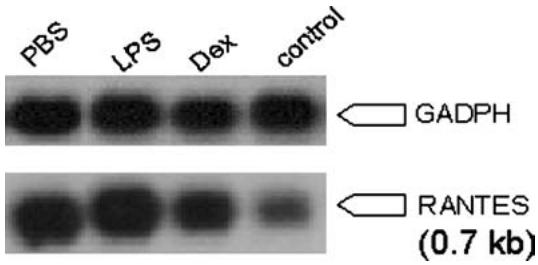

Fig. 5. RANTES mRNA expression in $KMnO_4$–induced calcified granuloma tissue in rats assessed by Northern blot analysis. mRNA from granuloma tissue i.p.-treated with LPS or Dex and PBS-treated control. A total of 10 μg total RNA was used for the Northern blot. Expression of RANTES transcripts increased in granuloma tissue of rats treated *in vivo* with LPS. Dex treatment induced mRNA RANTES reduction. Blots were stripped and rehybridized with a cDNA probe for the GAPDH RNA to adjust for small variations in RNA loading and transfer to the membrane. This blot is representative of three independent experiments with similar results.

RANTES Generation by Minced Calcified Granuloma Tissue In Vitro

The results in Table 2 show the amount of RANTES detected in the supernatant of minced granuloma tissue treated or not with LPS *in vitro* and incubated overnight (18 hours). The granuloma tissue was enucleated from rats after 7 days of treatment with LPS, Dex, or PBS (control). *In vitro* exposure of the minced tissue to LPS (100 ng/mL) strongly increased RANTES (pg/mL/100 mg) in all samples, whereas in granuloma tissue from animals treated with Dex i.p. no significant change was observed in RANTES concentration.

Table 1. RANTES release (pg/mL/100 mg homogenized tissue) in the supernatant of homogenized calcified granuloma tissue obtained by s.c. injection of $KMnO_4$ (see Materials and Methods) determined by ELISA

Treatment (*in vivo*)	No. animals	First set of experiments	$P < 0.05$	Δ %	Second set of experiments	$P < 0.05$	Δ %
PBS (control)	12	305 ± 80	*		262 ± 75	*	
LPS	12	1,443 ± 193	0.05	+373.1%	1,523 ± 175	0.05	+481.3%
Dex	12	186 ± 20	0.05	−60.9%	167 ± 15	0.05	−63.7%

Eighteen hours before sacrifice, animals were treated with LPS (6 μg/200 μL bolus injection) or Dex (300 μg/200 μL bolus injection), while control animals were treated with PBS. Data are expressed as mean ± SD. Values represent two sets of four experiments in triplicate. *P* values (Student's *t*-test) were calculated by comparing control (*) with LPS or Dex

Table 2. RANTES concentration (pg/mL/100 mg), determined by ELISA, in conditioned medium from minced granuloma tissue induced in rats with $KMnO_4$ and i.p.-treated *in vivo* with LPS, Dex, or PBS (as described in Materials and Methods)

Treatment *in vivo*	No. animals	−LPS *in vitro*	+LPS *in vitro*	$P < 0.05$	Δ %
PBS (control)	12	455 ± 82	1,430 ± 275	0.05	+214.28%
LPS	12	1,680 ± 395	3,560 ± 195	0.01	+111.9%
Dex	12	337 ± 20	570 ± 150	NS	+69.1%

LPS (100 ng/mL) or nothing was added *in vitro* in all specimens from previously i.p.-treated animals. Data are expressed as mean ± SD. *P* values (Student's *t*-test) were calculated by comparing the nil (−LPS) groups with the *in vitro* LPS-treated groups. NS, not significant

Table 3. ELISA determination of RANTES in the serum of animals with $KMnO_4$-induced granulomas treated for 18 hours with LPS, Dex, or PBS (control) before sacrifice

	No. animals	Serum RANTES (pg/mL)	$P < 0.05$	Δ %
PBS (control)	12	32 ± 8	*	
LPS	12	136 ± 6	0.01	+325%
Dex	12	14 ± 4	0.05	−43.7%

Data are expressed as mean ± SD. *P* values (Student's *t*-test) were calculated by comparing control (*) with LPS or Dex

Blood Serum RANTES Determination after Exposure to LPS or Dex

After 7 days from administration of $KMnO_4$, serum was collected from granuloma-bearing rats and analyzed for the presence of RANTES by ELISA. Table 3 shows that RANTES (pg/mL) was produced in higher quantity ($P < 0.01$) in LPS-treated animals, whereas when Dex was used, levels were lower ($P < 0.05$) compared to controls (PBS). Rats were treated i.p. 18 hours before sacrifice.

Discussion

The chemokine ligands for the CCR5 receptor, including RANTES/CCL5, can be induced in various activated cell types, such as lymphocytes, macrophages, dyskeratosis congenita (DCs), and endothelial cells. Recently, RANTES and other CCR5 ligands have been proposed to have a role in the positive regulation of Th1-cell polarization [36–38]. However, the *in vivo* role of RANTES is not well characterized, and its role in leukocyte activation needs to be confirmed. A relationship between RANTES and Th1-type cellular response was proposed since its neutralization determines a reduction of mycobacterial antigen-elicited granulomatous inflammation of the lung. Also, differential expression and cross-regulatory function of RANTES during mycobacterial (type 1) and schistosomal (type 2) antigen-elicited granulomatous inflammation have been observed [39].

Augmented expression of RANTES has been associated with many pathological inflammatory states, such as allogeneic transplant rejection, atherosclerosis, allergic disorders, glomerulonephritis, endometriosis, Alzheimer's disease, cancer, and arthritis [40–44].

Polymorphisms of chemokine genes, including RANTES, and chemokine-receptor counterpart genes (CCR5, CCR2, CX[3]CR1) have been associated with sensitivity to HIV infection and the natural course of the disease. Serum RANTES concentration is elevated in ovarian cancer patients and correlates with the severity of disease [45, 46]. RANTES is overexpressed in rheumatoid arthritis synovial fluid, suggesting that it can be a key mediator influencing the intensity and composition of cellular infiltration in joints affected by inflammatory arthritis [47, 48]. RANTES and other CC chemokines are suggested to play a direct role in the destructive phase of rheumatoid arthritis, participating in the modulation of migration, proliferation, and matrix metalloproteinase production through a mechanism involving intracytosolic calcium influx [49–51].

Recent studies *in vitro* show that RANTES is secreted by osteoclasts and promotes osteoclast growth and

survival in an autocrine/paracrine fashion. Moreover, osteoclast-derived RANTES induces osteoblast chemotaxis. Elevated calcium has been found to stimulate RANTES production by osteoclasts and osteoblasts [52]. Increased levels of RANTES were found in whole blood and the joints of the adjuvant-induced arthritis Lewis rat model of rheumatoid arthritis. Blocking of RANTES by specific antibodies ameliorated symptoms as much as indomethacin [53].

The nature of the relation between osteoarthritis and the various forms of calcium crystals that are found within osteoarthritic joints is debated. Arthroscopic assessment of patients suggests that the combined absence of chondrocalcinosis and identifiable crystals of synovial fluid may not be adequate to exclude clinically relevant crystalline deposition and inflammation [54].

Our study shows for the first time an *in vivo* causal effect of high local tissue concentration of RANTES, calcium crystal accumulation, and chronic inflammation and that this degenerative process can be enhanced by LPS and limited by administration of the antinflammatory compound Dex. Our results demonstrate that RANTES plays an important role in the inflammatory process. Therefore, we emphasize the relevance of chemokine RANTES and its receptors as potential therapeutic targets in inflammatory diseases of the joints.

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