Immunology 1997 **92** 300–306

Augmentation of monocyte chemotactic protein-1 and mRNA transcript in chronic inflammatory states induced by potassium permanganate ($KMnO_4$) *in vivo*

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SUMMARY

Monocyte chemotactic protein-1 (MCP-1) is a proinflammatory cytokine that attracts and activates specific types of leucocytes. The purpose of this work was to analyse the generation of MCP-1 and mRNA transcript in a model of chronic inflammation using a granulomatous tissue induced by potassium permanganate ($KMnO_4$; water soluble crystals). The data presented here shows that MCP-1 is generated in granuloma tissue and its level was strongly increased by i.p. injections of lipopolysaccharide (LPS) and inhibited in rats treated with injections of dexamethasone, 18 hr before the animals were killed. In histological studies LPS and dexamethasone increased and decreased, respectively, the recruitment of mononuclear cells in the granuloma tissue compared with the control granulomas from phosphate-buffered saline (PBS)-treated animals. Reverse transcriptase–polymerase chain reaction (RT–PCR) was used for mRNA extraction and cDNA synthesis. mRNA MCP-1 was significasntly produced in the granuloma tissue of untreated animals, an effect increased by LPS and inhibited by dexamethasone, compared with the controls. Moreover, MCP-1 protein was found in the supernatant from homogenized granuloma tissues and the levels of MCP-1 were higher in the LPS-treated animals, while they were lower in the dexamethasone group, compared with the granulomas from the PBS-treated groups (control). The generation of MCP-1 was also found in minced granuloma tissue incubated for 18 hr (overnight) from treated (LPS or dexamethasone) and untreated (PBS) rats. When LPS was added *in vitro* for 18 hr to the controls and treated animals the production of MCP-1 was further increased except in the dexamethasone group ($P > 0{\cdot}05$). Analysing blood serum from LPS, dexamethasone or PBS-treated rats, we found that MCP-1 was also present. The level was higher in the LPS group and lower in the dexamethasone group, compared with the control (PBS). In these studies we show for the first time that MCP-1 transcript and translation is generated in chronic experimental inflammatory tissue, an effect inhibited by dexamethasone.

INTRODUCTION

Monocyte chemotactic protein-1 (MCP-1) is a small (8000–10 000 MW) protein and a prototype member of the C-C chemokine-β subfamily, purified from different sources with chemoattractant and activator properties.[1–3] In contrast to chemokine-α proteins, chemokine-β proteins tend to attract and activate monocytes and lymphocytes at sub-nanomolar concentrations. To understand the role of the proinflammatory cytokine MCP-1 in disease, investigators have studied the complexity of intracellular signalling and the various biological activities of this chemokine.[4] In some inflammatory states, such as asthma, where the macrophage has a central role in the pathology of the disease, high levels of MCP-1 has been observed *in vivo*.[3–4]

Chronic inflammation is characterized by tissue infiltration with a wide variety of inflammatory cells. These cells play important roles in inflammation and natural immunity, and function to eliminate microbes and dead tissue.[5] The migration of leukocytic inflammatory cells is the central event in the inflammatory response and it is presumably based on the existence of locally produced chemotactic factors,[6–8] such as members of the C-X-C and C-C chemokine groups. Moreover, the response of leucocytes to tissue injury and infection can be potentially harmful and contribute to the pathogenesis of many diseases and inflammatory disorders.

Recently it has been reported that MCP-1 is a potent

Received 26 March 1997; revised 25 May 1997; accepted 4 June 1997.

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proinflammatory protein and plays a critical role in acute inflammation and allergic diseases.[2–4] We have shown in a recent report that MCP-1, after 4 hr induced steady-state levels of histidine decarboxylase (HDC) mRNA in basophilic cells *in vitro* and *in vivo*, mediating acute inflammation.[7–8] Moreover, MCP-1 can recruit and activate basophilic cells to the site of inflammation.[4]

Monocytes from peripheral blood accumulate at sites of chronic inflammation and delayed-type hypersensitivity reactions, an effect probably due to β intercrine subfamily members, which have been reported to be selective chemoattractants for human monocytes, rather than neutrophils.[1–4] We have recently reported that tissue granuloma formation induced by potassium permanganate in the mouse is infiltrated by numerous mononuclear cells, probably recruited by chemotactic factors[9] and thus mimicking the chronic inflammatory response. In spite of the many reports published on the effects of MCP-1 on mononuclear cells, there are relatively few studies involving MCP-1 in chronic inflammatory states *in vivo*. With these observations in mind, it was pertinent to study the generation of MCP-1 in chronic inflamed tissue induced by potassium permanganate ($KMnO_4$) and stimulated by lipopolysaccharide (LPS) or inhibited by dexamethasone in treated and untreated animals.

MATERIALS AND METHODS

The experimental animals used were male Wistar rats (Stefano Morini, Reggio Emilia, Italy), homogeneous for weight (250–280 g) and age (≈ 3 months), and raised under the same environmental and feeding conditions. All rats received four dorsal subcutaneous injections (0·2 ml) of a saturated solution (1 : 40 dilution) of potassium permanganate crystals.[10–13] Seven days after the $KMnO_4$ injections, the rats were anaesthetized and killed. Eighteen hours before the rats were killed, they were divided into groups of three (A, B, and C), each group having three controls (no treatment) receiving phosphate-buffered saline (PBS; 200 μl) intraperitoneally, three animals treated with LPS 6 μg/200 μl i.p bolus injection/rat and dexamethasone 300 μg/200 μl i.p bolus injection/rat. The granuloma formed at the injection sites were carefully measured, calculating the mean of the major diameters, expressed in millimetres. After the rapid enucleation of the fresh granulomas, the average weight, expressed in grams, was calculated for each group. Then some of the fresh granulomas were examined histologically (group A) and others were analysed for mRNA extraction and cDNA synthesis (group B), while still others were homogenized in cold PBS (group C). Aliquots were then tested by enzyme-linked immunosorbent assay (ELISA) for MCP-1 release. In another set of experiments (group D) granuloma tissues were enucleated, finely minced and vortexed in RPMI-1640 and incubated in the same medium plus 5% fetal calf serum in a test tube (Falcon, Milan, Italy) overnight (18 hr). Aliquots were then tested by ELISA for MCP-1 release.

Optical microscopy studies

The granulomatous tissues of animals induced with $KMnO_4$ (1 : 40 saturated crystal solution) after 7 days were enucleated and immersed in a fixative solution. Slides were prepared with sections of the tissue and coloured with Giemsa/May–Grunwald and analysed under an optical microscope (×20) (ID02, Carl Zeiss, Oberkochen, Germany). The mononuclear cells were counted in the optic field using a grating size of 5 × 5 mm. The cells were counted in the granuloma tissues that were treated, 18 hr before killing, with PBS, LPS or dexamethasone.

Granuloma tissue homogenation

Granulomatous tissue were surgically isolated and placed in ice-cold PBS and homogenized at 200 ***g*** for 5 min., resuspended in 2 ml ice-cold PBS, and homogenized by using a Polytron (Brinkmann Instruments, Westbury, NY) for 30 s. During these procedures, the tissues were kept in ice. The homogenized tissues were centrifuged at 1000 ***g***, 4° for 10 min, and supernatants were analysed for MCP-1 production.

Minced granuloma tissue treatments

A precise amount of minced granuloma tissue in RPMI-1640 was placed in plastic test tubes (Falcon) for each granuloma from LPS or dexamethasone-treated, or not treated (PBS) animals and exposed only to the vehicle or exogenous LPS for an overnight (18 hr) incubation. Afterwards, the test tubes were centrifuged at 1000 ***g*** at 4° for 10 min and the levels of MCP-1 in the culture supernatants were determined by a specific ELISA.

Blood serum tested for MCP-1 production after exposure to LPS or dexamethasone

Peripheral blood was obtained from intracardiac puncture of anaesthetized rats, from 7 day $KMnO_4$ treatments and collected in polypropylene tubes (Falcon). The animals were treated with LPS or dexamethasone 18 hr before sacrified. Serum was collected by whole blood centrifugation (650 ***g*** for 10 min) after clot formation. Tests were performed immediately after collection. The serum was then collected and tested for MCP-1 generation by ELISA.

MCP-1 ELISA

Antigenic MCP-1 was quantified using a solid-phase sandwich ELISA for mouse MCP-1 (Bio-Source, Camarillo, CA).[14] A monoclonal antibody specific for MCP-1 was coated onto the wells of flat-bottomed 96-well microtitre plates. Supernatants or standards were added and incubated for 2 hr at 37°. During the first incubation, the MCP-1 antigen binds to the immobilized antibody on one site. After washing, a biotinylated polyclonal antibody specific for MCP-1 was added. During the second incubation, this antibody binds the immobilized MCP-1 captured during the first incubation. After removal of excess biotinylated antibody, streptavidin–peroxidase was added. After a third incubation and washing to remove all of the unbound enzyme, a substrate solution was added which was acted upon by the bound enzyme to produce colour. The intensity of the coloured product is directly proportional to the concentration of MCP-1 present in the original specimen.

mRNA extraction and cDNA synthesis

Poly A mRNA was extracted using a purification system kit (Pharmacia Biotech, Milan, Italy). In brief, about 10 million cells were dissolved in a solution containing guanidinium thiocyanate 4 M and *N*-lauroylsarcosine in order to preserve the RNA. The solution was placed in oligo(dt)-cellulose at

25 μg/ml suspended in a storage buffer containing 0·15% Kathon CG (Pharmacia LKB, Cologno Monzese (MI), Italy). After several washes in salt buffers containing 10 mM Tris–HCl (pH 7·4), 1 mM ethylenediaminetetra-acetic acid (EDTA), 0·5 M NaCl or 0·1 M NaCl in the last two washes, the oligo(dt)-cellulose containing mRNA was placed in filter columns and the mRNA was eluted in warm Tris–HCl 10 mM and precipitated in chilled 95% ethanol overnight. After centrifugation, the pellet was dissolved in 14 μl of diethylpolycarbonate-treated sterile water and quantified by spectrophotometric analysis. 0·5 μg of mRNA was transcribed in cDNA incubating it with 200 U of superscript reverse transcriptase (Gibco BRL, Milan, Italy) and 50 ng of random examers (Gibco BRL).

Reverse transcriptase-polymerase chain reaction (RT–PCR) amplification

cDNA was amplified with 2·5 U Taq polymerase (Perkin Elmer Cetus, Milan, Italy) using 1·5 pM of each primer specific for MCP-1 and glucose-3-phosphate dehydrogenase (G3PDH). MCP-1 and G3PDH primers were purchased from Clonetech Laboratory (Palo Alto, CA). Each sample was divided in half, one part was used for the cytokine under investigation, the other half for the G3PDH for semiquantitative analysis. RT–PCR was conducted for MCP-1 with the following protocol: 1 min. at 94°, 1 min. at 58° for MCP-1 (62° for G3PDH), 1 min. at 72°. Thirty cycles were used for MCP-1 and 25 cycles for G3PDH. The linear range of signal strength for MCP-1 mRNA was determined by performing titration for cDNA and cycle numbers to obtain non-saturated PCR reactions for each cytokine. Five millilitres of amplified products were electrophoretically separated in 2% agarose gel containing ethidium bromide and finally analysed for molecular size. The following controls were used: cDNA without primers; normal muscle tissue.

Signals were analysed by software Bio-profile (Vilber Lourmat, Nice, France), and semi-quantitative analysis was possible comparing the amplified product signals with the G3PDH signal.

Statistical analyses

Data from different experiments were combined and reported as the mean ± SD. Student's *t*-test for independent means was used to provide a statistical analyses ($P > 0.05$ was considered as not significant).

RESULTS

Induction of granuloma by $KMnO_4$

The $KMnO_4$-induced granuloma formation in the rat was assayed for chronic inflammation. In Table 1 we show the mean ± SD of four experiments performed in triplicate. Each rat received a subcutaneous injection 200 μl of a saturated solution of $KMnO_4$ crystals. After 7 days we calculated the mean ± SD of the major diameters (in mm), the mean ± SD of the weights of 12 granulomas in the three rats (for each experiment). Eighteen hours before the rats were killed, they were divided into groups of three, each group having three controls (no treatment) receiving PBS (200 μl) intraperitoneally, three animals treated with LPS 6 μg/200 μl i.p. bolus injection/rat and dexamethasone 300 μg/200 μl i.p. bolus injection/rat. The size and weight of all granulomas from LPS treated animals were significantly higher ($P < 0.05$) than the untreated animals (control PBS), while rats treated with dexamethasone were lower ($P < 0.05$) than the controls. The increase was obtained in LPS-treatment, which was ≈51·7% (mean of major diameters) and 115·5% (mean weights of granulomas). The inhibition was obtained in dexamethasone-treatment, which was, respectively, 47·1% in the diameters and 48·7% for the weight, with respect to controls.

Mononuclear cell recruitment in granuloma tissue from rats treated i.p. with PBS, LPS, and dexamethasone

After granuloma induction by $KMnO_4$, the rats were i.p. treated with PBS, LPS, and dexamethasone 18 hr before killing. The animals were divided into groups of three, each group having three controls (no treatment) receiving PBS (200 μl); three animals treated with LPS 6 μg/200 μl and dexamethasone 300 μg/200 μl. In order to evaluate the inflammatory state of the granuloma tissue histological studies were performed in three experiments in triplicate. Figure 1 shows a significant increase ($P < 0.05$) of mononuclear cells in a biopsy of granuloma tissue treated with LPS (b) and a significant decrease ($P < 0.05$) of mononuclear cells in the biopsy treated with dexamethasone (c) compared to the control (a) (PBS). This is a representative experiment. This effect was observed in all animals. The effect of dexamethasone treatment suggests that this steroidal antiinflammatory drug inhibited the number of mononuclear cell leucocyte accumulation, while LPS increased it.

Table 1. Mean weights (g) and mean of the major diameters (mm) of granulomas formed after 7 days from injections of $KMnO_4$ in rats

$KMnO_4$ Treatment	No. of Experiment	Mean of the major diameters (mm) of granulomas	$P <$	Δ%	Mean (g) of granulomas	$P <$	Δ%
Control	4	17·2 ± 1·6	(*)	(−)	0·0877 ± 0·02	(*)	(−)
LPS	4	26·1 ± 1·7	(0·05)	(+51·7)	0·189 ± 0·05	(0·05)	(+115·5)
Dex	4	9·1 ± 1·8	(0·05)	(−47·1)	0·045 ± 0·02	(0·05)	(−48·7)

These values represent the mean of the measured diameters and the net weights of the granuloma in triplicate in four separate experiments. Each animal received four dorsal injections of $KMnO_4$ (1:40 saturated solution) and treated or not with intraperitoneally administered LPS (6 μg/200 μl bolus injection/rat) or dexamethasome 300 μg/200 μl bolus injection/rat), injected 18 hr before the animals were sacrificed. *P* values (Student's *t*-test are calculated by comparing control (*) with LPS or dexamethasone.

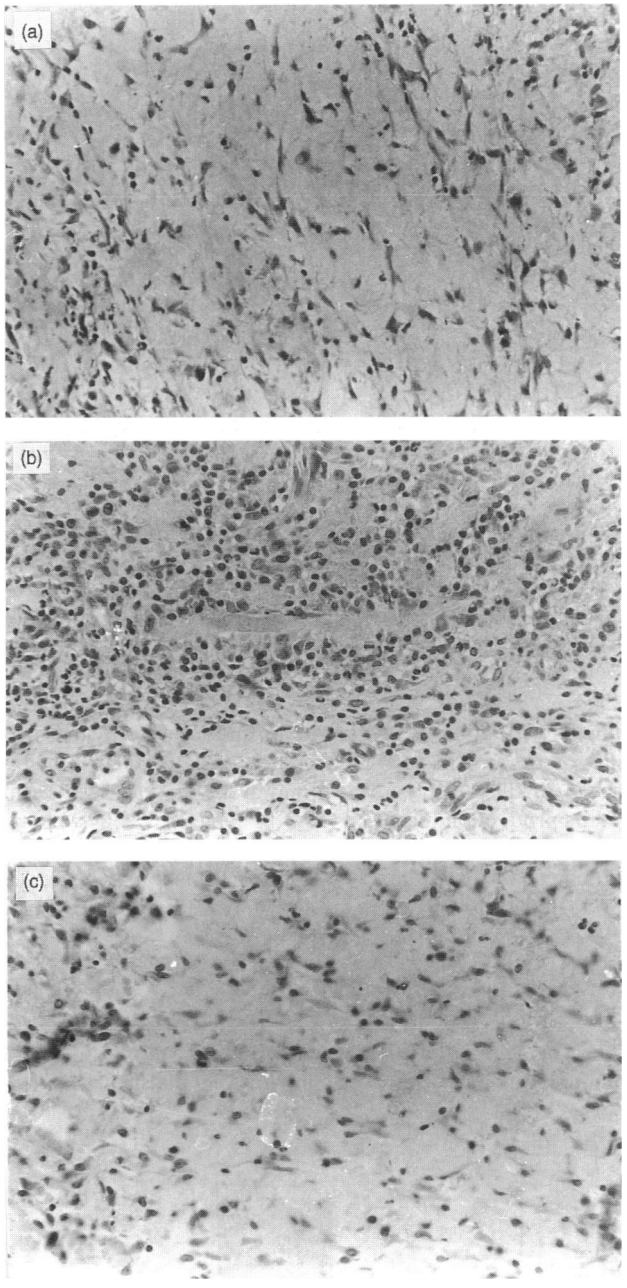

Figure 1. Microscopic appearance (14×) of rat granuloma tissue produced by 200 µl injection of $KMnO_4$ saturated-crystal solution diluted 1:40. (a) rat granuloma from untreated animal; (b) rat granuloma from LPS (6 µg/200 µl bolus injection/rat) treated animal; (c) rat granuloma from dexamethasone (300 µg/200 µl bolus injectin/rat) treated animal. The animals were injected with the compounds 18 hr before killing. (b) shows a marked increase ($P<0{\cdot}01$) in number of mononuclear cells compared to (a), control; while (c) shows the reduction ($P<0{\cdot}05$) in number of mononuclar cells. *P*-values (Student's *t*-test) are calculated by comparing control (*) with LPS or dexamethasone.

MCP-1 generation in the granuloma tissue

Because MCP-1 has been presented as a potent chemotactic factor for mononuclear cells and a mediator of inflammatory state, in these studies we evaluated the generation of MCP-1 from granuloma inflamed tissue. Here we show the amount of MCP-1 (pg/ml/100 mg tissue) from treated and untreated homogenized granuloma tissue from animals treated with LPS or dexamethasone. Each set of experiments was the mean ± SD of three experiments performed in triplicate. The detected MCP-1 was enhanced by LPS (1150–720 pg/ml, first and second set of experiments, respectively; while it was inhibited by dexamethasone (190–60 pg/ml first and second experiments), compared to the PBS (310–280 pg/ml) control (Fig. 2).

Generation of MCP-1 mRNA levels in granuloma tissue *in vivo*

Because MCP-1 has been presented as a proinflammatory compound, to evaluate this effect more fully a RT–PCR analysis was determined for MCP-1 mRNA in chronic inflammatory states. In Fig. 3a steady-state levels of MCP-1 mRNA in the granuloma tissue control were significantly generated in all experiments. In animals treated with LPS, MCP-1-specific mRNA was significantly increased compared to the control, while in the presence of dexamethasone MCP-1 mRNA was significantly inhibited, compared to the control. MCP-1 mRNA was undetectable at 1 hr while was detectable at 2 hr (data not shown) and reached maximum levels at 4 hr. At 6 hr mRNA levels were also induced and were not different that 4 hr. Normal muscle tissue biopsied from the same dorsal area of normal rats expressed undetectable MCP-1 mRNA levels. Figure 3b shows on the *y*-axis the ratio of MCP-1/G3PDH mRNA measured by a computerized system (software Bioprofile, Vilber Lourmat, France); while the treatment is represented on the *x*-axis. The data presented herein quantify the value of MCP-1 mRNA in the granuloma tissue.

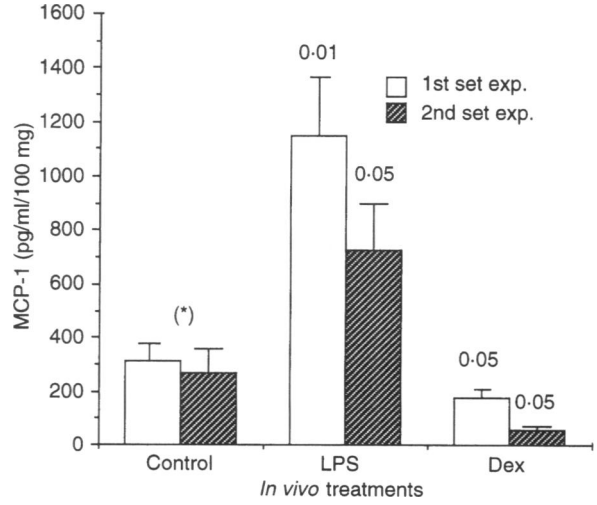

Figure. 2. Mean ± SD of MCP-1 protein generation in supernatants from homogenized granuloma tissue. 18 hr before sacrifice, the animals were treated with LPS (6 µg/200 µl bolus injection/rat) and dexamethasone (300 µg/200 µl bolus injectin/rat) and the control animals were treated with PBS. The values represent two sets of four experiments in triplicate. *P*-values (Student's *t*-test) are calculated by comparing control (*) with LPS or dexamethasone.

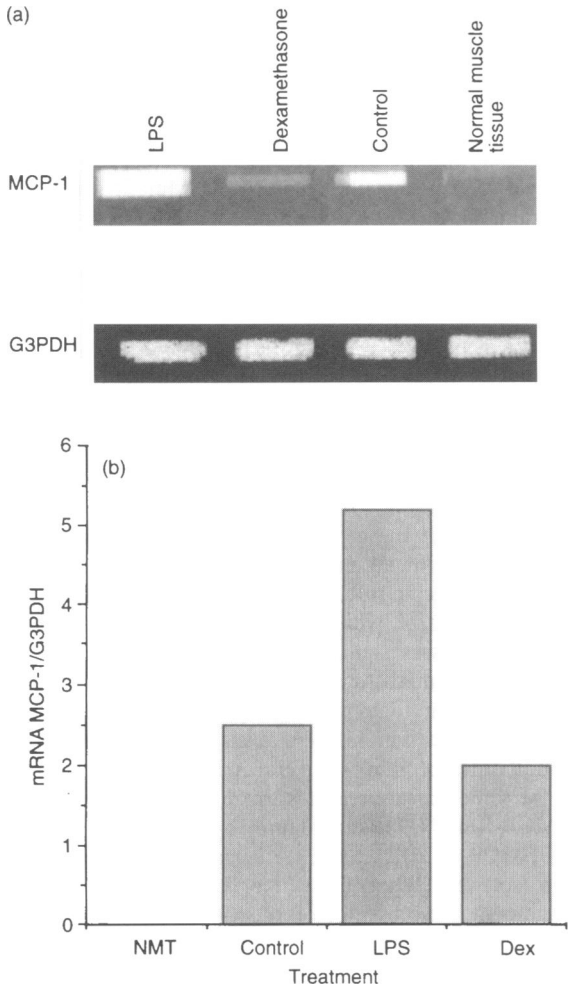

Figure 3. (a) Generation of MCP-1 mRNA and expression in untreated and treated granuloma tissue induced subcutaneously by $KMnO_4$. Normal muscle tissue was removed in the dorsal area of a normal rat (where granuloma was not induced). In (b) we show the ratio of MCP-1 mRNA and G3PDH and the percentage of MCP-1 mRNA expression in the granuloma tissue. This is a representative experiment of three.

Augmentation of MCP-1 by LPS after an overnight incubation from minced granuloma tissue from treated (LPS and dexamethasone) and untreated rats

In these experiments, the minced granuloma tissues from treated and untreated rats were all incubated with LPS to determine the potential effect of LPS to further induce the production of MCP-1 (Fig. 4). In this figure LPS enhanced the production of MCP-1 in the control, in the LPS-treated animals; while it was not significant in the dexamethasone group. This data confirms the capacity of LPS to induce MCP-1.

Serum levels of MCP-1

It has been previously reported that LPS is capable of inducing MCP-1 in leucocytes and this effect is inhibited by glucocorticoids.[15–16] Here we measured the generation of MCP-1 in the serum of the rat after 7 days of granuloma formation. The

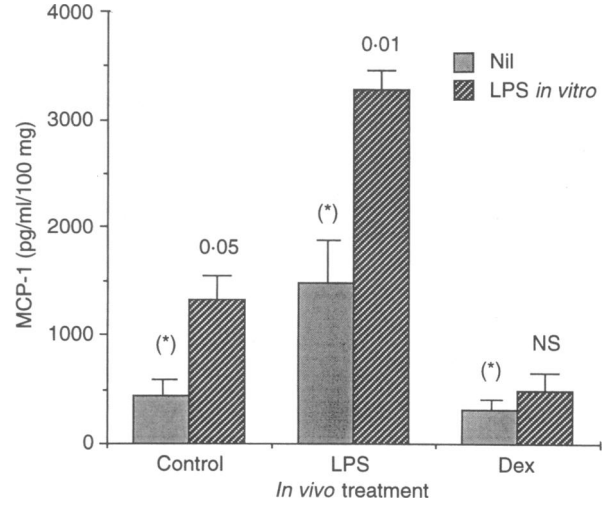

Figure 4. Mean ± SD of MCP-1 protein from minced granuloma tissue from i.p.-treated (LPS or dexamethasone) and i.p.-untreated (PBS) *in vivo* animals (light grey column). The striped column represents the addition of LPS in all *in vitro* specimens from previously i.p. treated and untreated animals. *P*-values (Student's *t*-test) are calculated by comparing the light grey column (*) with the striped columns.

animals with induced granuloma formation were treated with LPS or dexamethasone, or not treated (PBS) 18 hr before they were killed. MCP-1 was found in the untreated rat serum and its production increased (59·5 ± 3·5 pg/ml, $P < 0{\cdot}01$) in the animals treated with LPS (6 μg/200 μl bolus injection/rat) compared to the controls (16 ± 6 pg/ml); while decreased (5·7 ± 2·2 pg/ml, $P < 0{\cdot}05$) in the dexamethasone treatment (300 μg/200 μl bolus injection/rat) (Fig. 5).

DISCUSSION

The property of MCP-1 to recruit mononuclear cells to the inflammatory site has been previously published.[2–4] These

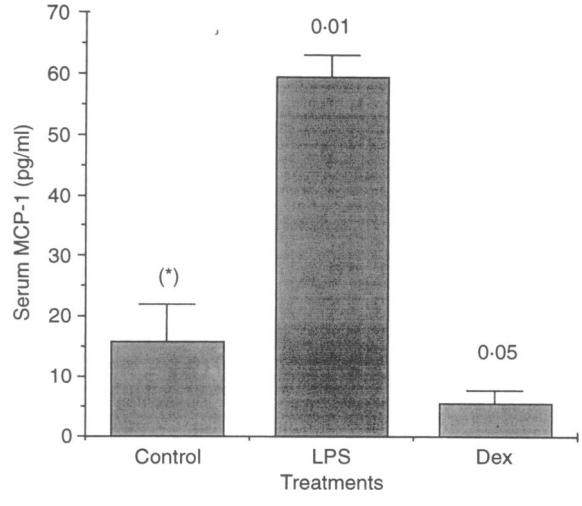

Figure 5. The values represent the amounts ± SD in pg/ml of MCP-1 protein from blood serum of animals treated 18 hr before killing with LPS and dexamethasone, or untreated (control). *P*-values (Student's *t*-test) are calculated by comparing control (*) with LPS or dexamethasone.

reports suggest that MCP-1 could play a significant role in inflammatory states. In view of these observations, we studied the generation of MCP-1 in a chronic inflammatory state consisting of an experimentally formed granulomatous tissue after 7 days, produced by potassium permanganate ($KMnO_4$) water-soluble crystals, a good and valid model for the study of *in vivo* chronic inflammation.[10–13]

In these studies we have found that MCP-1 is generated in granuloma tissue. The levels of MCP-1 were strongly increased by i.p. injections of LPS and inhibited in animals treated with injections of dexamethasone 18 hr before the animals were killed. Moreover, in histological studies LPS and dexamethasone increased and decreased, respectively, the recruitment of mononuclear cells in the granuloma tissue, compared to the control granulomas from untreated rats. Because local production of MCP-1 was found, it was of interest to determine by RT–PCR if MCP-1 mRNA was detectable in the granuloma tissue formed. mRNA MCP-1 was significantly produced in the granuloma tissue of untreated animals. The fact that MCP-1 was found in the blood serum indicates that this cytokine mediates not only the local inflammatory response[17–19] but also can affect the animals systemically. The generation of MCP-1 protein and mRNA transcripts, associated with LPS-stimulated granuloma tissue and mononuclear cell accumulation suggests that MCP-1 is released in the inflammatory site and may play a role in the activation and in the recruitment of mononuclear cells at sites of induction of chronic inflammation. In addition, the production of MCP-1 protein and mRNA transcript increased by LPS emphasizes that inflammation and cell recruitment are part of the response to tissue injury by $KMnO_4$. The ability of granuloma tissue to express MCP-1 mRNA increased by LPS stimulation was dependent on *de novo* protein synthesis. Our findings suggest that a distinct pathway of activation exists for the production of important monocyte-derived chemotaxin, MCP-1. We propose that inflammatory injury stimulates mononuclear cells to produce chemokines that attract other phagocytic cells from the parenchima and circulation. Such mononuclear cells may have the capacity to generate cytokines that further modify the leucocyte activity.

Our data demonstrate, for the first time, that MCP-1 expression and secretion is generated in chronic inflammatory tissues induced by potassium permanganate and that this effect is increased by LPS. The mechanism whereby LPS acts in increasing MCP-1 is not clear. However, it is possible that LPS may increase the production of other cytokines, such as interleukin (IL)-1 and tumour necrosis factor-α (TNF-α), which are classic proinflammatory proteins.[20–21]

Another hypothesis is that LPS itself, acts directly as a proinflammatory compound inducing MCP-1, because the polysaccharide portion of LPS is covalently linked with a lipid moiety called lipid A, which is the endotoxic principle of LPS causing inflammation.[22–25] Moreover, the release of chemoattractant and vasoconstrictive arachidonic acid compounds, such as leukotriene B_4 (LTB_4), in the inflammatory site, can also play an important role in immunofunction of formed granuloma tissue.[9,27]

In our studies to inhibit the biosynthesis of MCP-1 we used dexamethasone (a glucocorticoid which exerts its effect by binding to intracellular receptors) which directly modulates gene transcription by targeting specific DNA binding sites called positive GRE,[26] which may represent an important signal for transcription. Non-steroidal antiinflammatory agents were not used since it has been previously reported that they fail to decrease MCP-1 transcript and protein levels.[15]

The modulation of chemokines can henceforth affect the outcome of the inflammatory response.[27–30] Recent studies show that MCP-1 is present in fibrotic tissue including pulmonary fibrosis and atherosclerosis and plays a critical role in the wound-healing response,[31] demonstrating that chemokines can play an important role in initiating tissue repair process by chemoattracting immune cells into a wound site.[2,32]

The results presented here are in accordance with Taub *et al.* who demonstrated that MCP-1, 2, and 3 are inflammatory mediators stimulating the directional migration of human $CD4^+$ and CD8 T-cell clones, as well as monocytes.[19] Our studies contribute to understanding the mechanisms by which mononuclear cells profoundly affect chronic inflammatory responses *in vivo* and suggest that the antagonist(s) of MCP-1 may have inhibitory biological effects on inflammatory conditions. Moreover, the present data describes an additional biological activity of MCP-1, suggesting that this cytokine may have an important effect on chronic inflammatory diseases mediated by immunoactivated mononuclear cells.

ACKNOWLEDGMENTS

This work was supported by the Italian Ministry of Education 1996. We wish to thank Dr Alfredo Grilli of the Biology Division for the technical assistance and for preparing the figures.

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