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Constitutive Expression of Macrophage-Inflammatory Protein 2 (MIP-2) mRNA in Bone Marrow Gives Rise to Peripheral Neutrophils with Preformed MIP-2 Protein

Sigrid P. Matzer, Tobias Baumann, Nicholas W. Lukacs, Martin Röllinghoff, and H. Ulrich Beuscher

Macrophage-inflammatory protein 2 (MIP-2) is a major CXC chemokine involved in the migration of polymorphonuclear neutrophils (PMNs) to sites of inflammation. Although cell culture experiments have identified different cell types that can produce MIP-2, the cellular sources in vivo are not clearly defined. By using immunohistochemical staining and analysis of chemokine mRNA expression, the present study aimed to localize cells producing MIP-2 in tissues of normal mice and mice challenged with Yersinia enterocolitica. The results showed a constitutive expression of MIP-2 mRNA in bone marrow (BM) of normal mice, but not in other organs such as spleen, lung, or liver. MIP-2 protein was found in all organs tested but it was exclusively associated with PMNs that stained positive with the cell surface marker Gr-1. Bacterial infection caused a 5-fold increase in the number of MIP-2-positive PMNs recruited to spleens concomitant with a strong increase of splenic MIP-2 mRNA. This correlated well with a 3-fold loss of MIP-2-producing cells in BM. Because MIP-2 mRNA expression in PMNs was increased after stimulation with TNF, the results indicate that newly recruited PMNs can supplement their MIP-2 content through TNF-stimulated transcription. Together, the data imply a constitutive production of MIP-2 by a subset of PMNs in BM and argue for the possibility of a rapid mobilization of MIP-2 through its storage in circulating PMNs. The Journal of Immunology, 2001, 167: 4635–4643.

Polymorphonuclear neutrophils (PMNs) represent the body’s first line of defense and hence are rapidly recruited from the circulation to sites of infection. In addition to their microbicidal functions, evidence has accumulated to show that PMNs are an important source of cytokine mediators, including IL-1, IL-3, IL-6, IL-12, TNF, TGF-β, and IL-1 receptor antagonist (1, 2). Some severe clinical disorders, e.g., inflammatory bowel disease (3) and glomerulonephritis (4), are associated with infiltration of PMNs and elevated tissue levels of proinflammatory cytokines such as IL-1. Because treatment with IL-1R antagonist reduced the severity of the inflammation in inflammatory bowel disease (5), these and other studies suggest that PMN and PMN-derived cytokines could play a role in the pathogenesis of inflammatory diseases.

Migration and extravascular accumulation of PMNs is a multi-step process and requires a series of regulatory signals that include expression and activation of adhesion molecules (6) as well as the generation of chemotactic gradients by cells of the extravascular compartment (4). Chemotaxis of immune cells is mediated by a large group of small basic proteins, commonly termed chemokines (7, 8). They are categorized into four families C, CC, CXC, and CX3C, based on the position of one or two conserved cysteine residues in the amino (N) terminus. Migration of PMNs is mediated by members of the growing CXC chemokine family, including human IL-8 (9) (CXCL8; Refs. 10 and 11), the human growth-related oncogenes (GRO-α–GRO-CL1–3) and their murine homologues macrophage-inflammatory protein (MIP) 2/CXCL1 and platelet-derived growth factor-inducible chemokine KC (KC/ CXCL2) (12, 13). The potent neutrophil chemotactic activity of each of the three chemokines requires the presence of a glutamate-leucine-arginine (ELR) motif near the N terminus, and preceding the first conserved cysteine residue. Both MIP-2 and KC were shown to bind to the murine CXCR2, which is abundantly expressed on granulocytes and as recently shown on NKT cells (14, 15). When neutrophils from CXCR2 knockout mice were exposed to MIP-2 or KC, no migration was observed and specific blocking of CXCR2 using a chemokine antagonist resulted in inhibition of neutrophil recruitment in response to TNF, IL-1, or LPS, supporting the view that CXCR2 is the principal receptor for CXC chemokines on neutrophils (16, 17).

MIP-2 has been shown to be one of the major inducible chemokines with the ability to attract neutrophils to the site of inflammation (18, 19), although other mediators such as C5a need to be considered (20). Expression of MIP-2 mRNA has been observed in response to microbial infection, injection of LPS, and stimulation of cells with proinflammatory mediators such as IL-1 and TNF (21–23). Several cell types including macrophages (24), epithelial cells (25), bone marrow (BM) endothelial cells (25), astrocytes (26), and mast cells (27) have been identified as cellular sources of MIP-2. In addition, like one of its functional homologues, such as

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*Institute for Clinical Microbiology, Immunology, and Hygiene, University of Erlangen, Erlangen, Germany; †Departments of Pathology and Internal Medicine, University of Michigan Medical School, Ann Arbor, MI 48109

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S.P.M. and T.B. contributed equally to this study.

Address correspondence and reprint requests to Prof. Dr. H. Ulrich Beuscher, Institute for Clinical Microbiology, Immunology, and Hygiene, University of Erlangen, Wasserturmstrasse 3, 91054 Erlangen, Germany. E-mail address: beuscher@microbio.med.uni-erlangen.de

Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; MIP, macrophage-inflammatory protein; BM, bone marrow; MOI, multiplicity of infection; DAPI, 4-diamidino-2-phenylindol-di-hypochloride; MCP-1, monocyte chemotactic protein 1; IP-10, IFN-γ-inducible protein 10; TCA-3, T cell activation gene 3; PBL, peripheral blood leukocyte.

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human IL-8, MIP-2 has been suggested to be produced by neutrophils (28, 29), although available data to support this conclusion are rather limited. The concept of MIP-2 production by neutrophils, however, is intriguing because neutrophils are the first cells recruited to sites of infection and the release of chemokines by these cells may have an important impact on the development of early immune responses. For example, the early release of MIP-2 could promote recruitment of more neutrophils or even modulate the type of leukocyte infiltration. In view of these considerations, the present study aimed to characterize the production of MIP-2 in response to an acute bacterial infection. As a model we used the i.p. infection of mice with the Gram-negative Yersinia enterocolitica (30). In humans and rodents, this enteric pathogen causes inflammatory diseases that range from gastroenteritis to ileitis and lymphadenitis. Replication of Y. enterocolitica takes place in lymphoid tissues and depends on the expression of plasmid-encoded, secreted virulence proteins to evade the immune system. The host response to Yersinia infection is characterized by a massive infiltration of granulocytes and monocytes into infected tissues and application of Y. enterocolitica into the peritoneal cavity of mice leads to abscess formation in spleen, liver, and the peritoneal wall. Our results suggest that Gr-1-positive granulocytes rapidly accumulate in infected spleens and a subpopulation of these cells displays MIP-2 protein. In the absence of an ongoing infection, these MIP-2-positive granulocytes were found in spleens and BM; however, MIP-2 mRNA was only detectable in BM, but not in spleens. These results suggest that MIP-2 is produced by PMNs during maturation in BM and then stored.

Materials and Methods

Bacteria

The avirulent strain (NCTC 10598), here named O:8-, and the isogenic virulent strain (NCTC 10938), here named O:8+, of Y. enterocolitica O:8 were obtained from the National Collection of Type Cultures (Central Public Health Laboratory, London, U.K.). Bacteria were routinely grown overnight in defined trypton yeast extract glucose medium at 26°C (31). For infection, Yersinia cultures grown overnight were diluted 1/20 in Luria-Bertani medium and incubated at 37°C for 2 h; bacteria were harvested and washed twice with PBS (Biochrom, Berlin, Germany).

Experimental infection of mice

Female BALB/c mice (6–10 wk of age) were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany). Mice were infected i.p. with 1 ml of Yersinia suspension containing 5 × 10⁴ CFU. Mice were sacrificed at different time points (0–6 days) after infection. Spleens, lungs, BM, and blood cells were harvested. For determination of bacterial content, tissues were homogenized in saline containing 0.1% deoxycholic acid (Sigma, Deisenhofen, Germany) using a Potter homogenizer. Appropriate dilutions of the homogenates were plated onto Yersinia-selective agar containing the selective supplement SR 109 (Oxoid, Basingstoke, U.K.). After 2 days of incubation at room temperature, the numbers of CFU were counted.

Cells and cell culture

Peritoneal exudate cells were prepared from peritoneal cavities of mice 3 days after a single i.p. injection of 1 ml of 10% thioglycollate (Difco, Offenbach, Germany) and cultured in 6-well-plates at a density of 4 × 10⁶ cells/well (21). The culture medium Clicks/RPMI 1640 was supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Biochrom), and 10% FCS (Sigma). After 3 h of incubation (37°C, 5% CO₂, 95% humidity), nonadherent cells were washed off. Subsequently, the peritoneal macrophages were stimulated for 2 h with LPS (10 µg/ml) or with Y. enterocolitica O:8+ at a multiplicity of infection (MOI) of 10. Casein-elicited granulocytes were prepared from peritoneal cavities of mice 3 h after a single i.p. injection of 2 ml of 0.2% casein (Sigma) (32) and cultured in 6-well plates as described for preparation of peritoneal exudate cells. After 4 h of incubation (37°C, 5% CO₂, 95% humidity), the granulocyte containing nonadherent cell fraction was recovered. Subsequently, granulocytes were incubated in 12-well plates at a density of 1 × 10⁶ cells/well with Y. enterocolitica O:8+ at a MOI of 10. After 1 h of incubation, the cell-free supernatants were sterile filtered and subjected to a MIP-2 ELISA.

Single-cell suspensions of spleens were prepared as described previously (31). Briefly, spleens were excised and gently passed through a net. BM cells were obtained by flushing femurs and tibiae with culture medium. Contaminating erythrocytes were removed by suspending the spleenic and BM cells in NH₄Cl solution (168 mM) for 10 min at room temperature. To obtain peripheral blood leukocytes (PBL), heparinized blood was diluted at a ratio of 2:1 with PBS and applied onto a NIM-2 two-step density gradient (Cardinal Associates, Santa Fe, NM) according to the manufacturer’s instructions to gently remove erythrocytes. After centrifugation (1000 × g, 25 min, 20°C) cells were collected from both the upper and interphase of the gradient, washed with PBS, and resuspended in culture medium. In some experiments, BM was treated similarly. BM-derived granulocytes were prepared using the same procedure as described for blood leukocytes, except that granulocytes were collected only from the interphase.

To prepare cytospin preparations, single-cell suspensions of spleens, BM, and PBL were adjusted to a density of 1 × 10⁶ cells/ml and placed onto microscopic slides by centrifugation (21 × g, 2 min) in a cytocriscent (Shandon, Pittsburgh, PA). The slides were dried overnight at room temperature and fixed in acetone (10 min, −20°C) for subsequent immunostaining.

FACS analysis

Spleenic single-cell suspensions (1 × 10⁶ cells/ml) were preincubated with Fc-Block (1.25 µg/10⁶ cells; BD Biosciences, San Jose, CA) for 10 min followed by PE-conjugated rat anti-murine Gr-1 Ab (clone RB6-8C5; BD Biosciences). The Gr-1 (Ly-6G) Ag is expressed on granulocytes including neutrophils (PMNs) as well as eosinophils although the neutrophils represent the vast majority of Gr-1-positive cells in the periphery. A further discrimination between neutrophils and eosinophils was not approached because the neutrophil-specific mAb 7/4 (Serotec, Oxford, U.K.) does not react with neutrophils from BALB/c mice sufficiently as described by the manufacturer (33). After incubation for 45 min, cells were thoroughly washed with PBS/1% FCS. All steps were conducted at 4°C. Samples were analyzed by flow cytometry using a FACSCalibur and CellQuest software (BD Biosciences).

Immunohistochemistry

Preparation of frozen tissue sections and immunoperoxidase staining were performed as described elsewhere (34). Briefly, cryostat sections (5 µm) and cytospin preparations were incubated overnight with appropriate dilutions of either rat anti-murine Gr-1 Ab (clone RB6-8C5; BD Biosciences) or rabbit anti-murine MIP-2 Ab purchased from PeproTech (London, U.K.) or prepared as described previously (35). To block staining, recombiant MIP-2 (100 µg/ml; PeproTech) was preincubated with rabbit anti-murine MIP-2 for 8 h. After thoroughly washing, the slides were incubated with biotin-conjugated secondary stage Abs: mouse anti-rat IgG and donkey anti-rabbit IgG (Dianova, Hamburg, Germany). Streptavidin-biotinylated HRP kits were used according to the suppliers’ recommendations (Dako, Hamburg, Germany) and the peroxidase was visualized with 3-aminio-9-ethylcarbazole (Sigma). Nuclei were stained using Mayer’s hematoxylin (Dr. K. Hollborn and Söhne, Leipzig, Germany). For immunofluorescence, double-staining tissue sections and cytospin preparations were blocked in PBS containing 0.1% saponin (Sigma) and 20% FCS and incubated simultaneously with both primary Abs described above. After extensive washing with PBS/0.1% Tween 20, FITC-conjugated donkey anti-rabbit IgG (BD Biosciences) and Rhodamine Red X-conjugated donkey anti-rat IgG (Dianova) secondary Abs were applied for 45 min at room temperature. Cytoospin preparations were additionally counterstained with the DNA stain 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 µg/ml PBS; Boehringer Mannheim, Mannheim, Germany). Slides were mounted in Mowiol (Hoechst, Frankfurt, Germany) containing 1,4-diazabicyclo[2,2,2]octane (Sigma) as an antifading reagent. Cells and tissue sections were examined by using an Axiopt microscope (Zeiss, Oberkochen, Germany) equipped with appropriate filters to gate FITC, Rhodamine Red X and DAPI fluorescence. Photographs were acquired and processed using a Spot camera and Metaview software (Diagnostic Instruments, Burroughs, MI).

ELISA

MIP-2 protein levels in serum, spleen, or cell culture supernatants were quantitated using the murine Quantikine MIP-2 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Spleens were excised, immediately snap frozen in liquid nitrogen, and
stored at −70°C until homogenization in PBS containing complete protease inhibitor mixture (Roche Diagnostic Systems, Mannheim, Germany) using a Potter homogenizer. ELISA results from splenic homogenates were normalized to sample weight to correct for differences in sample size.

**RNase protection assay**

Total RNA was prepared from frozen tissues or single-cell suspensions by using the guanidine-thiocyanate extraction procedure followed by acid-phenol extraction (36). RNase protection assays were performed using Ribotomo assay kits purchased from BD Biosciences according to the manufacturer’s instructions. Briefly, radiolabeled antisense RNA probes were generated from the murine cDNA template sets mCK-5 and mCR-6 containing lymphotactin/XCL1, RANTES/CCL5, eotaxin/CCL11, MIP-1β/CCL4, MIP-1α/CCL3, MIP-2, IFN-γ-inducible protein 10 (IP-10)/CXCL10, monocyte chemotactant protein 1 (MCP-1)/CCL2, T cell activation gene 3 (TCA-3)/CCL1, and CXCR2, CXCR4, Burkitt lymphoma receptor 1/CXCR5, respectively) and hybridized to target RNA. ssRNA was digested with RNase and protected probes were separated on 5% polyacrylamide sequencing gels. Gels were dried and visualized by autoradiography or using a phosphoimager (Fuji, Elmsford, NY). Quantification was performed using AIDA software (Ray Test, Straubenhardt, Germany).

**Reverse transcription and semiquantitative PCR**

The cDNA was synthesized from 2 μg of total RNA using 2.5 μM oligo(dT)12–18, 1 mM dNTP, 12 U Rnaseguard (Amersham Pharmacia Upjohn, Freiburg, Germany), 200 U Superscript II reverse transcriptase, 80 mM DTT (Life Technologies, Karlsruhe, Germany), and 1 mM MgCl2 (Peqlab, Freiburg, Germany), 200 U Superscript II reverse transcriptase, 80 mM DTT (Life Technologies, Karlsruhe, Germany), and 1 mM MgCl2 (Peqlab, Erlangen, Germany) to a final volume of 25 μl at 42°C for 60 min. For MIP-2 and β-actin-specific PCR, equal amounts of sample cDNA were amplified in 25-μl reaction volumes containing 1 mM dNTP (Amersham Pharmacia Upjohn), 1 U Taq DNA polymerase (Peqlab), and 20 μM primers during 35 cycles (30 s denaturation, 94°C; 30 s annealing, 58°C for β-actin, 55°C for MIP-2; 30 s polymerization, 72°C) with an Omnigene temperature cycler (Hybaid, Ashford, U.K.) to allow quantitative considerations. The primers for β-actin and MIP-2 were purchased from Amersham Pharmacia Upjohn and MWG Biotech (Ebersberg, Germany), respectively, and were as follows: β-actin: sense primer, 5′-CACCCGCCACAGTTCGCCA-3′; antisense primer, 3′-CAGGTCCCGGCCAGGCGAT-5′ (amplified fragment 574 bp); MIP-2: sense primer, 5′-CCACTCTCAAGGGCGGTCA-3′; antisense primer, 3′-CCCCTATCCCAGCCTTTCA-5′ (amplified fragment 515 bp). PCR samples were separated on 1% agarose gels and visualized by ethidium bromide staining and photographed on an ImageMaster VDS (Amersham Pharmacia Upjohn).

**Results**

**Preferential induction of MIP-2 mRNA expression in Yersinia-infected mice**

To establish the kinetics of induction of chemokine genes, mice were challenged i.p. with virulent *Y. enterocolitica* O:8+, and total RNA was prepared from spleens and lungs on days 0–6 postinfection. RNase protection assays were used to compare mRNA levels of lymphotactin, RANTES, eotaxin, MIP-1α, MIP-1β, MIP-2, IP-10, MCP-1, and TCA3. As shown in Fig. 1A, in the spleen low levels of transcripts were detectable for eotaxin, MIP-1α, MIP-1β, IP-10, and MCP-1 from days 2 to 6. Constitutive mRNA expression was only found for RANTES, the levels of which slightly increased by day 2. In comparison, only MIP-2 mRNA, though not detectable until day 2, preferentially increased during infection, i.e., days 4–6. Similar results were obtained when analyzing the expression of MIP-2 in lungs (Fig. 1A). In

![FIGURE 1. Induction of murine chemokine mRNA expression in response to infection with *Y. enterocolitica* O:8+ (Y.e. O:8+). Total RNA was extracted from lung or splenic tissue (A and B) at the indicated times postinfection and subjected to RNase protection assay. Representative data are shown. A. Kinetics of chemokine mRNA expression after infection (i.p.) with virulent Y.e. O:8+. B. Comparison of MIP-2 mRNA expression in spleen after infection with avirulent Y.e. O:8− and virulent Y.e. O:8+. Control mice (c) were left untreated. C. Cultured peritoneal macrophages were stimulated with Y.e. O:8+, LPS, or left untreated (c). Total RNA was prepared after 4 h of incubation and subjected to RNase protection assay. Ln, Lymphotactin.](http://www.jimmunol.org/Download)
comparison, MIP-2 mRNA was not found in spleens of mice challenged with the avirulent strain Y. enterocolitica O:8/H11002 (Fig. 1B), indicating that MIP-2 expression is associated with the disease. To further assess the potency of Yersiniae as stimulants of chemokine expression, macrophage cultures were incubated in parallel with virulent bacteria or LPS. Subsequent RNase protection assays of total RNA revealed both stimuli as equally potent for up-regulation of RANTES, MIP-1α/H9251, MIP-1β/H9252, MIP-2, IP-10, and MCP-1 mRNA expression. No transcripts were detected for eotaxin and TCA3 (Fig. 1C). The data show that the pattern of chemokine mRNA induction is much more restricted during infection of mice as compared with infection of macrophages in tissue culture.

Kinetics of leukocyte accumulation in Yersinia-infected spleens

To correlate MIP-2 expression with leukocyte migration, we next assessed the kinetics of appearance of Gr-1-positive granulocytes (granulocytesGr-1/H11001) in Yersinia-infected spleens. Results (Fig. 2A) obtained by flow cytometric analysis show that the relative numbers of granulocytes gradually increased over time, reaching a plateau on days 5–6 after infection. In addition, RNase protection assays were performed with total splenic mRNA to determine the expression of CXCR2, the established neutrophil receptor for MIP-2. As compared with mRNA levels from uninfected mice, there was a 24-fold increase in CXCR2 mRNA on day 6 after infection (Fig. 2B). Also, bacterial burden increased over time such that by days 4 and 5 postinfection a 100-fold excess of the inoculated CFU could be recovered from infected spleens (Fig. 2C). The data indicate that recruitment of leukocytes into infected spleens is temporally coordinated with the appearance of MIP-2 mRNA shown in Fig. 1A.

Yersinia infection leads to enhanced MIP-2 protein levels

To determine whether Yersinia infection causes the release of MIP-2, systemic as well as local MIP-2 protein levels were deter-
mined. Therefore, the amount of MIP-2 protein was measured using an ELISA. Serum and splenic tissue samples of infected and uninfected mice were collected 6 days after infection. The data summarized in Fig. 3 show that infection caused elevated levels of MIP-2 in serum as well as in splenic homogenates. Interestingly, the levels of MIP-2 in those specimen correlated with the relative number of bacteria (Fig. 3C). The data therefore suggest that the induction of MIP-2 synthesis relates to the severity of the infection.

**MIP-2 protein is found in granulocytes**

To define the cellular sources of MIP-2, consecutive tissue sections of infected spleens were subjected to immunoperoxidase staining. The anti-Gr-1 Ab identified a discrete abscess area (Fig. 4A) in which only a few cells stained positive with the anti-MIP-2 antiserum (Fig. 4B). Immunochemistry demonstrated that MIP-2 stained positively with granulocytes Gr-1++ (yellow), indicating that MIP-2 is produced by a subset of recruited granulocytes Gr-1++ (Fig. 4C). Apparently no other cell types were identified to synthesize MIP-2. In controls, immunostaining for MIP-2 was blocked by an excess (100 µg/ml) of recombinant MIP-2 (data not shown), thus confirming the specificity of MIP-2 immunodetection. Finally, immunoperoxidase staining (Fig. 4D) and immunofluorescence double staining (Fig. 4E) revealed MIP-2 protein in granulocytes Gr-1++ of spleens of uninfected mice as well. However, when counting MIP-2-positive granulocytes (granulocytes Gr-1++/MIP-2++) in splenic cell suspensions of control and diseased mice, it became evident that infection with *Y. enterocolitica* caused a 4-fold increase in the number of granulocytes Gr-1++/MIP-2++. These data argue for a production of MIP-2 by newly recruited

*FIGURE 4.* Analysis of the phenotype of MIP-2-producing cells in spleens. Consecutive tissue sections of infected spleens were subjected to immunoperoxidase staining (A and B) or immunofluorescence double staining (C) with Abs to Gr-1, MIP-2, or a combination of both (C) that were recognized with the secondary Abs anti-rat-Rhodamine Red X and anti-rabbit-FITC, respectively. Data are representative for two experiments. Original magnification, ×400. Single-cell suspensions of uninfected mice were subjected to immunoperoxidase staining with anti-MIP-2 Abs or to immunofluorescence double staining with anti-MIP-2 and anti-Gr-1 Abs as described above (D and E).
granulocytes \textsuperscript{Gr-1+}, but also raise the issue of a constitutive MIP-2 expression.

**Constitutive expression of MIP-2 mRNA occurs in BM but not spleen cells**

To address the discrepancy between detection of MIP-2 protein (Fig. 4) and lack of MIP-2 mRNA (Fig. 1A) in spleens of uninfected mice, expression of MIP-2 mRNA was analyzed by PCR. No MIP-2 mRNA was found in spleens (Fig. 5A), but it was readily detectable in BM (Fig. 5B). Immunofluorescence double staining of BM cytospin preparations further demonstrated that MIP-2 protein colocalized with granulocytes \textsuperscript{Gr-1+} (Fig. 5C). In addition, morphological analysis revealed MIP-2 staining in two cell forms with either ring-shaped nuclei, namely, PMN-like ring cells (Fig. 5E) or PMN/neutrophils (Fig. 5D). The data indicate that MIP-2 gene expression occurs constitutively in BM and further imply that MIP-2 can exist in peripheral granulocytes \textsuperscript{Gr-1+} as a prestored chemokine in the absence of detectable MIP-2 mRNA transcription.

**Granulocytes \textsuperscript{Gr-1+}/MIP-2\textsuperscript{+} are depleted from BM during Yersinia infection**

Because PMN are normally short-lived, the fate of granulocytes \textsuperscript{Gr-1+}/MIP-2\textsuperscript{+} was analyzed in response to infection with \textit{Y. enterocolitica}. In control mice, high numbers of granulocytes \textsuperscript{Gr-1+}/MIP-2\textsuperscript{+} were found in BM, concomitant with low numbers in peripheral blood and spleens. During infection, however, the amount of granulocytes \textsuperscript{Gr-1+}/MIP-2\textsuperscript{+} markedly decreased in BM (Fig. 6A). The loss of granulocytes \textsuperscript{Gr-1+}/MIP-2\textsuperscript{+} in BM of infected mice was specific in that no significant decrease was observed in the total number of granulocytes \textsuperscript{Gr-1+} (Fig. 6B). The data indicate that mobilization of leukocytes during infection yields a selective depletion of granulocytes \textsuperscript{Gr-1+}/MIP-2\textsuperscript{+} from BM. In addition, data summarized in Table I show that \textit{Yersinia} infection produced a change in the proportion of granulocytes \textsuperscript{Gr-1+}/MIP-2\textsuperscript{+} in BM such that the relative amount of PMN/neutrophils \textsuperscript{MIP-2+} increased, while there was a decrease of PMN-like ring cells \textsuperscript{MIP-2-}. The data argue in favor of a subset of granulocytes committed for MIP-2 production.

**TNF and IL-1 increase MIP-2 mRNA expression in BM cells**

To gain insights into the regulatory mechanisms of MIP-2 production, primary cultures of BM were stimulated with IL-1 and TNF. For each stimulus, one set of cultures was analyzed for MIP-2 mRNA expression by PCR and a second set was used for analysis of MIP-2 protein synthesis by immunostaining. Both cytokines were found to strongly increase the relative amounts of MIP-2 mRNA as compared with untreated cultures (Fig. 7A). However, none of the two cytokines was able to significantly change the numbers of cells with detectable MIP-2 protein after 2 or 24 h of expoision (Fig. 7B), suggesting that transcription and translation of MIP-2 are independently regulated. Alternatively, it is possible that MIP-2 mRNA is increased in cells already committed for MIP-2 production.

Finally, we were interested in determining whether \textit{Yersinia} bacteria are able to stimulate the release of MIP-2 from granulocytes. For this purpose, casein-elicited granulocytes were prepared.
FIGURE 7. Induction of MIP-2 mRNA expression in BM. Single-cell suspensions of BM either unstimulated (c) or stimulated for 2 h with IL-1 (100 ng/ml) or TNF (100 ng/ml) were subjected to semiquantitative RT-PCR with primers for MIP-2 and actin (A). The results show that IL-1 and TNF increase MIP-2 mRNA levels. After 2 or 24 h, stimulated BM cells were additionally subjected to immunostaining for MIP-2 and anti-Gr-1 (fluorescence double staining using cytospin preparations) and counterstained with hematoxylin. For each specimen, a minimum of 300 MIP-2-positive cells/group were analyzed in two separate experiments. The results indicate that granulocytes Gr-1+/MIP-2+ are associated with granulocytes. As determined by immunofluorescence microscopy, detection of MIP-2 mRNA strictly correlated with the cell surface marker Gr-1, which is primarily expressed on mature granulocytes (39). In BM, ~45% of all cells were recognized by the Gr-1 Ab, whereas ~15% of the cells stained positive for both Gr-1 and MIP-2. This suggests that within the population of granulocytes Gr-1+/MIP-2+, only a subset of cells is committed to produce MIP-2. In addition, a morphological classification based on the criteria provided by Biermann et al. (40) indicates that granulocytes Gr-1+/MIP-2+ can be subdivided into two subsets, namely, PMN-like ring cells and PMN/neutrophils (Table I). One characteristic of PMN maturation includes the acquisition of granules and granule products (41). Hence, a likely explanation for the existence of subsets of granulocytes Gr-1+/MIP-2+ are differences in timing of granule protein synthesis during maturation of PMN precursors. In cell culture experiments as well as in animals, other cell types such as macrophages, epithelial cells (23), and mast cells (27) have been described as a source of MIP-2. In control experiments, we observed also that anti-MIP-2 antisera recognized MIP-2 in LPS-stimulated macrophages (data not from the peritoneal cavity and incubated for 1 h with 10 MOI of Y. enterocolitica. Analysis of the supernatants by ELISA revealed MIP-2 levels between 5 and 10 pg/ml (data not shown), supporting the notion that Yersiniae are able to trigger the release of preformed MIP-2 from granulocytes.

### Discussion

In this study, we have shown that MIP-2 is produced by neutrophils and that its expression is regulated, in part, during marrow maturation. The data confirm and extend previous observations indicating that MIP-2 is associated with granulocytes (28, 29). Several lines of evidence suggest a model in which MIP-2 mRNA is constitutively expressed in BM by a subpopulation of neutrophils which are released into the periphery with a preformed MIP-2 protein. First, under physiologic conditions, detectable levels of MIP-2 mRNA were found exclusively in BM (Fig. 5B) and synthesis of MIP-2 protein in BM was associated with granulocytes Gr-1+/MIP-2+ (Fig. 5C). Second, in the absence of MIP-2 mRNA (Fig. 5A), the MIP-2 protein was found in splenic granulocytes Gr-1+ of uninfected mice (Fig. 4E) as well as in circulating blood leukocytes (data not shown). Similar to our results with MIP-2, IL-6 protein was previously found in circulating murine PMN devoid of IL-6 mRNA, while at the same time expression of IL-6 mRNA was readily detectable in BM cells (37). In addition, murine neutrophils have recently been shown to contain intracellular stores of IL-12 (38). The data point to a common regulatory mechanism through which intracellular cytokines are acquired as part of the normal developmental program of neutrophils.

As determined by immunofluorescence microscopy, detection of MIP-2 strictly correlated with the cell surface marker Gr-1, which is primarily expressed on mature granulocytes (39). In BM, ~45% of all cells were recognized by the Gr-1 Ab, whereas ~15% of the cells stained positive for both Gr-1 and MIP-2. This suggests that within the population of granulocytes Gr-1+/MIP-2+ only a subset of cells is committed to produce MIP-2. In addition, a morphological classification based on the criteria provided by Biermann et al. (40) indicates that granulocytes Gr-1+/MIP-2+ can be subdivided into two subsets, namely, PMN-like ring cells and PMN/neutrophils (Table I). One characteristic of PMN maturation includes the acquisition of granules and granule products (41). Hence, a likely explanation for the existence of subsets of granulocytes Gr-1+/MIP-2+ are differences in timing of granule protein synthesis during maturation of PMN precursors. In cell culture experiments as well as in animals, other cell types such as macrophages, epithelial cells (23), and mast cells (27) have been described as a source of MIP-2. In control experiments, we observed also that anti-MIP-2 antisera recognized MIP-2 in LPS-stimulated macrophages (data not shown).
shown), consistent with the detection of MIP-2 mRNA expression (Fig. 1). However, throughout the entire study, immunostaining of tissue specimens from infected mice as well as control mice failed to specifically identify MIP-2-synthesizing cells other than granulocytes\textsuperscript{Gr-1} \textsuperscript{MIP-2}. It cannot be excluded, however, that the levels of MIP-2 protein are increased in granulocytes\textsuperscript{Gr-1} \textsuperscript{MIP-2} due to its storage in granules, which in turn may facilitate immunorecognition of neutrophil-associated MIP-2.

Infection of mice with \textit{Y. enterocolitica} caused a preferential accumulation of MIP-2 mRNA in infected tissue, as compared with mRNA levels of other chemokines such as MIP-1\textalpha, MIP-1\beta, RANTES, MCP-1, and IP-10 (Fig. 1). In contrast, no such preferential induction of MIP-2 mRNA was observed when macrophage cultures were infected with \textit{Y. enterocolitica}. Instead, each of the macrophage-derived chemokines was induced equally well by either LPS or the bacteria. This suggests that expression of MIP-2 in \textit{Yersinia} infection is selective. Underlying regulatory mechanisms may be provided by the release of proinflammatory mediators which like TNF have the potential to increase MIP-2 mRNA expression (27), whereas IL-10, one of the most potent anti-inflammatory agents, has been shown to suppress chemokine gene expression (21, 42). Alternatively, we propose that accumulation of MIP-2 mRNA at sites of infection is largely regulated through the influx of newly recruited neutrophils retaining the ability to express MIP-2 mRNA. Experimental support for this comes from kinetic experiments showing a temporal correlation between appearance of MIP-2 mRNA and neutrophil recruitment. Moreover, evidence that neutrophils display the ability to up-regulate MIP-2 expression in response to proinflammatory mediators comes from experiments showing that purified BM-derived granulocytes can be stimulated with TNF to increase MIP-2 mRNA (Fig. 7C). The constitutive expression of MIP-2 mRNA and protein found in BM-granulocytes (Figs. 7 and Fig. 5C) does not seem to be due to constitutive TNF expression in the BM, because granulocytes\textsuperscript{Gr-1} \textsuperscript{MIP-2} were found in BM of TNF-deficient mice (S. P. Matzer and H. U. Beusch, unpublished data).

In conclusion, the data of this study identify neutrophils as a major source of the chemokine MIP-2. They contribute to the concept of neutrophils as rapidly mobilized regulators of antimicrobial host responses (1) through preformed inflammatory as well as anti-inflammatory cytokines. Synthesis and release of preformed MIP-2 by neutrophils may have several biological implications: 1) peripheral neutrophils containing the preformed MIP-2 may act as alarm cells and regulate the early influx of PMNs to sites of infection and 2) in the setting of an ongoing acute inflammatory process neutrophil-derived MIP-2 may serve to amplify and perpetuate the recruitment of leukocytes from the circulation.

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References