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High Level Monocyte Chemoattractant Protein-1 Expression in Transgenic Mice Increases Their Susceptibility to Intracellular Pathogens

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We have constructed transgenic mice in which the mouse mammary tumor virus long terminal repeat controls the expression of murine monocyte chemoattractant protein-1 (MCP-1). Several independently derived lines of transgenic mice constitutively expressed MCP-1 protein in a variety of organs. Protein extracts from these organs had substantial in vitro monocyte chemoattractant activity that was neutralized by an anti-MCP-1 Ab, indicating that transgenic MCP-1 protein is biologically active. However, no transgenic mouse at any age displayed monocyte infiltrates in MCP-1-expressing organs. Two transgenic lines had circulating MCP-1 levels of 13 to 26 ng/ml, which is a concentration sufficient to induce maximal monocyte chemotaxis in vitro. These transgenic lines showed a 1 to 1.5 log greater sensitivity to infection with Listeria monocytogenes and Mycobacterium tuberculosis. A third transgenic line had lower serum levels of MCP-1 and was resistant to L. monocytogenes. The results suggest that this transgenic model is one of monocyte nonresponsiveness to locally produced MCP-1 due to either receptor desensitization or neutralization of a chemoattractant gradient by high systemic concentrations of MCP-1. Regardless of the mechanism, the data indicate that constitutively high levels of MCP-1 expression do not induce monocylic infiltrates, and that MCP-1 is involved in the host response to intracellular pathogens. The Journal of Immunology, 1995, 155: 4838–4843.

Leukocyte infiltration into tissues is the result of a complex set of molecularly distinguishable signals (1, 2). First, a circulating leukocyte must adhere loosely to the vascular endothelium, then in the presence of an appropriate stimulus, this adhesion becomes firm. Finally, the leukocyte will follow a concentration gradient of chemoattractant as it diapedeses to enter the inflammatory site. The fact that particular leukocyte subsets appear in an infiltrate to the exclusion of others suggests that the signals have target cell specificity. Although some specificity may be exerted through adhesion molecules with restricted patterns of leukocyte expression, there is increasing evidence that chemoattractant signals are also specific for leukocyte subsets.

Recently, a family of leukocyte-specific chemoattractants, called chemokines, has been described whose members may play important roles in generating inflammatory cell infiltrates (3, 4). Chemokines are low m.w. proteins that share a high degree of structural and genetic similarity, in particular four cysteines in conserved positions. Within the large chemokine family, two subfamilies can be distinguished based on the presence or the absence of an intervening amino acid between the two cysteines nearest the N-terminals. The former subfamily is denoted C-X-C, and the latter family is C-C. This structural distinction is reflected at a genetic level, since, to date, all of the genes that encode C-X-C chemokines map to the long arm of chromosome 4, while all of the C-C chemokine genes map to the long arm of chromosome 17.

There is also a partial functional distinction between the chemokine subfamilies. Although there are exceptions, most C-X-C chemokines, e.g., IL-8 and the GRO proteins, specifically attract neutrophils. In contrast, most C-C chemokines attract monocytes and memory T lymphocytes and, in some cases, eosinophils and basophils. Among the C-C chemokines, MCP-1 has been the most intensively studied. In vitro, MCP-1 attracts monocytes and memory T cells at subnanomolar concentrations (5–7) and is a potent stimulator of histamine release from mast cells (8, 9). Its expression has been documented in several diseases that are characterized by monocyte-rich inflammatory cell infiltrates, leading to the suggestion that MCP-1 expression may play a pathogenic role in diseases as diverse as rheumatoid arthritis and atherosclerosis (10–12). To date, however, it has been difficult to demonstrate conclusively that abnormally regulated MCP-1 expression is responsible for disease.

Part of the difficulty in ascribing pathogenetic roles to MCP-1 is uncertainty about its in vivo properties. In some animal models, intradermal injection of human MCP-1 produces an accumulation of monocytes at the inoculation site (13, 14). However, we have been unable to induce infiltrates in mouse skin in response to pure murine MCP-1 (15). One reason for the absence of infiltrates may have been an inability to supply high concentrations of MCP-1 at a local site for sufficiently long periods of time. To create a model that might mimic sustained MCP-1 synthesis during an inflammatory response, we constructed transgenic mice in which murine

*Abbreviations used in this paper: MCP-1, monocyte chemoattractant protein-1; MMTV-LTR, mouse mammary tumor virus long terminal repeat; CAPS, 3-cyclohexylaminol-1-propanesulfonic acid.
MCP-1 expression was controlled by a constitutive heterologous promoter. As it turned out, this was a poor model of physiologic MCP-1 release at local sites, since these mice produced high levels of MCP-1 in several organs throughout the animals’ lifetimes. Nonetheless, the phenotype of these transgenic mice is one of increased susceptibility to intracellular pathogens, and they are, thus, informative as a model of monocyte dysfunction due perhaps in part to desensitization to MCP-1. These results point to the involvement of MCP-1 in physiologic pathways that are required for the elimination of intracellular pathogens.

Materials and Methods

Transgenic mice

Genomic MCP-1 DNA (pGMJE-1) (16) was modified by substituting the XbaI to PvuII fragment from murine MCP-1 cDNA (pJeE-1) (16) for the XbaI to PvuII fragment in pGME-1. This manipulation removed genomic MCP-1 mRNA cap site and placed an EcoRI site at the 5’ end of the remaining MCP-1 DNA, a position +20 relative to the cap site. The EcoRI fragment from this construct (which contains genomic MCP-1 from +20 to +2006 bp beyond the polyadenylation signal) was placed downstream from the AvuI to BamHI fragment of the MMTV-LTR (17) to yield pMMJE +20, in which the MMTV-LTR cap site is 121 bp 5’ to the first nucleotide of modified genomic MCP-1. Thus, the final construct contains the MMTV-LTR promoter and its mRNA cap fused to genomic murine MCP-1 at a point 25 bp downstream from its own cap site. There were no manipulations of MCP-1’s coding sequences. Linearized transgene DNA, tritium of most of its plasmid vector sequences, was used to inject oocytes from FVB/N mice according to standard techniques (18). After transfer to foster mothers, progeny were tested for the presence of the transgene at 3 wk of age by Southern blot analysis. DNA was extracted from tail samples (19), and Southern blots were probed with a radiolabeled Naol-Hpal fragment derived from genomic murine MCP-1 DNA. Transgene copy number was estimated by comparing the intensity of a transgene signal (as determined by laser densitometry (Pharmacia, Piscataway, NJ)) to that of an endogenous gene signal. Mice were killed on various ages, and tissue sections were prepared for routine hematoxylin and eosin staining and for immunohistochemistry.

RNA analysis

Organs were harvested and homogenized in 1 ml of 4 M guanidine isothiocyanate, 25 mM sodium citrate (pH 7.0), and 100 mM 2-ME. RNA was purified from the homogenate by centrifugation through a cushion of 5.7 M cesium chloride, followed by precipitation with ethanol (20). Twenty micrograms of RNA was fractionated by electrophoresis through a 1.5% agarose gel containing 2.2 M formaldehyde and transferred to nylon-based membranes. Gels were stained with ethidium bromide and photographed to document the intensity of ribosomal RNA in each lane. Membranes were probed using standard techniques (21) with murine MCP-1 cDNA (16) radiolabeled with 32P deoxyribonucleotides by nick translation.

Immunoblot analysis

Organs were placed in 1 ml of 60 mM Tris-HCl (pH 6.8), 100 mM DTT, and 2% SDS; boiled for 5 min; sonicated; and clarified by centrifugation. Proteins were separated on 15% SDS-PAGE, then blotted to Immobilon membranes (Millipore Corp., Danvers, MA) in 10 mM CAPS (pH 11.0) and 10% methanol. MCP-1 protein was identified using rabbit anti-mouse MCP-1 serum as previously described (15, 22).

In vitro monocyte chemotaxis

Salivary glands from control or transgenic mice were placed in 1 ml of cold 50 mM Tris-HCl (pH 7.5)-0.2 mM EDTA and homogenized on ice. Extracts were clarified by centrifugation at 15,000 × g for 30 min, followed by a second centrifugation at 100,000 × g for 45 min. Serial dilutions were tested for human monocyte chemotactic activity using a 58-well microchemotaxis apparatus previously described (23). Concentration of monocyte chemotactic activity in monocyte chemotactic activity units per ml was defined as the inverse of the dilution demonstrating half-maximal activity (24). Immunoadsorption of MCP-1 from the extracts was performed using rabbit anti-mouse MCP-1 as previously described (25).

Listeria monocytogenes infection

Mice were injected i.p. with the indicated inocula of virulent L. monocytogenes (strain 1778), American Type Culture Collection designation 43251, obtained from Dr. Eric Pamer, Yale University, New Haven, CT. Mice were observed daily and killed if they were unable to right themselves or reach water.

Mycobacterium tuberculosis infection

Mice were injected i.v. with 8 × 104 CFU of strain H37Rv. Forty days after inoculation, mice were killed, and dilutions of homogenates from lungs, livers, and spleens were plated on enriched agar plates (Middlebrook 7H11, Difco Laboratories, Detroit, MI). Colonies were counted after 3 to 4 wk.

IL-1 challenge

Mice were anesthetized using inhaled methoxyflurane, and 200 ng of murine IL-1β (Genzyme, Boston, MA) was injected intradermally into the left footpad using a 27-gauge needle. An equivalent volume of sterile PBS was injected into the right footpad. Twenty-four and forty-eight hours after injection, mice were killed by CO2 inhalation, and footpads were processed for histologic analysis.

Murine MCP-1 ELISA

Plates (Nunc MaxiSorp “C” bottom 96-well plates, VWR, Boston, MA) were coated with 0.2 μg of MCP-1 capture Ab (18241D, PharMingen, San Diego, CA), diluted to 0.1 M NaHCO3, overnight at 4°C, then blocked with 3% BSA-PBS. Standards and samples were added in duplicate, and the plate was incubated for 4 h at room temperature. After washing, biotinylated detecting Ab (18272D, PharMingen) was added at 0.2 μg/well for 45 min, followed, after washing, by the addition of 0.25 μg/well avidin-peroxidase (Sigma Chemical Co., St. Louis, MO) for 30 min. The ABTS microwell peroxidase substrate system (Kirkegaard and Perry, Gaithersburg, MD) was used for detection. Results were read at 405 nm using a microtiter plate reader ( Molecular Devices, Sunnyvale, CA), and a standard curve was generated. The minimum detection limit for MCP-1 in the assay was 0.50 to 0.75 ng/ml.

Results

Expression of MCP-1 in transgenic mice

As described in Materials and Methods, we constructed a transgene in which murine genomic MCP-1 DNA was placed downstream from a fragment of the MMTV-LTR. Using Southern blotting, we identified 11 founder lines, most of which had one to five copies of the transgene per diploid genome, as determined by scanning analysis of Southern blot autoradiographs. However, one line, denoted Tg33, had at least 30 copies of the transgene, and this high copy number was stably inherited.

Of the 11 founder lines, 5 lines (Tg21C, Tg24, Tg25, Tg33, and Tg59) expressed transgenic mRNA that was distinguishable from wild-type mRNA by its increased size (data not shown) because MCP-1 mRNA is not expressed constitutively in any organ of wild-type FVB mice. Figure 1 shows expression of transgenic MCP-1 mRNA in four of these lines. The pattern of expression was consistent with the reported properties of the MMTV-LTR, namely high levels of expression in the salivary glands of three lines (17). Expression in other organs, in particular lung, also occurred in some lines. The pattern of expression in line Tg33 (the line with a high number of tandem insertions) was remarkable for its low levels of expression in salivary gland and high levels in kidney.

We documented MCP-1 protein expression in some of the organs that expressed transgene mRNA. Figure 2 shows an immunoblot analysis of salivary gland, lung, and testis extracts from a control mouse and three transgenic mice demonstrating high levels of the fully glycosylated forms of murine MCP-1 (15) in transgenic salivary glands. (MCP-1 protein expression was also documented in the salivary glands of the remaining two transgenic lines that expressed transgenic mRNA (data not shown).) Lower amounts of MCP-1 protein were expressed in lungs and testes of two of the three lines. We have never detected constitutive MCP-1 protein expression in any organ from nontransgenic mice. This analysis was performed on boiled extracts from transgenic organs.
to insure that material bound to connective tissue elements would be released. However, experiments on nondenatured extracts (next section) demonstrated that substantial amounts of MCP-1 were freely soluble.

Activity of transgenic MCP-1

The functional integrity of transgenic MCP-1 protein was determined by testing salivary gland extracts in a monocyte chemotaxis assay. (Figure 3, left) compares monocyte chemoattractant activity in an extract from a Tg24 salivary gland to activity in an extract from a nontransgenic littermate. The transgenic extract had over 8000 times the monocyte chemoattractant activity of the nontransgenic extract (22.2 U/mg total protein compared with 0.0026 U/mg total protein). Furthermore, immunoabsorption with an anti-MCP-1 Ab removed nearly 90% of the monocyte chemoattractant activity from the transgenic extract (Fig. 3, right).

Histologic and hematologic analysis of transgenic mice

We examined organs from transgenic mice, aged 2 to 52 wk. Despite the expression of high levels of biologically active MCP-1, there were no histologic abnormalities in any organ of any transgenic mouse that were not also present in nontransgenic littermates. In particular, we observed no monocyte infiltrates in any organs either by examination of routine hematoxylin and cosin sections or by immunohistochemical analysis using the murine monocyte/macrophage-specific Ab F4/80 (26).

A small proportion (<10%) of Tg33 mice developed a fatal disorder characterized by profound leukocytosis with extramedullary hematopoiesis and ulcerative proctocolitis. Some mice developed WBC counts of 100,000/μl, comprised almost entirely of mature neutrophils. The occurrence of this phenotype in a single line suggests that it may have been due to insertional mutagenesis, although this line also had the highest number of transgene copies and the highest levels of circulating MCP-1 (see below). The basis for this phenotype is still under investigation. All other transgenic lines had normal blood counts.

**In vivo challenges**

There are several possible explanations for the lack of monocyte infiltration in organs that constitutively expressed transgenic MCP-1. First, MCP-1 expression could be necessary, but not sufficient to elicit an infiltrate. To test this hypothesis, we administered varying doses of LPS or IL-4 to transgenic mice, but neither of these costimulators produced monocyte infiltration in expressing organs. Second, transgenic mice may be incapable of mounting any sort of inflammatory cell response. To test this, we challenged transgenic and nontransgenic mice by injection of murine IL-1β into the footpad. Figure 4 shows that transgenic and nontransgenic mice responded to IL-1β injection with cellular infiltrates of equal intensity, comprised of neutrophils and mononuclear cells.

Another explanation for the apparent absence of an abnormal phenotype is that transgenic mice secreted such high levels of MCP-1 that their circulating monocytes could not respond, because of either monocyte desensitization or neutralization of the chemoattractant concentration gradient. This model would predict measurable levels of MCP-1 in the blood of transgenic mice. In fact, Table 1 shows that Tg24 and Tg33 mice had serum levels of MCP-1 ranging from 13 to 26 ng/ml; however, a third line, Tg25, had only 4 ng/ml. As expected, control mice had less than 1.0 ng/ml. The levels of circulating MCP-1 in Tg24 and Tg33 mice are...
The Transgenic mice (except for the small proportion of Tg33 mice) are capable of mounting histologically normal foreign body responses to material in surgical wounds (data not shown). This suggests that their monocytes are not completely inactivated. However, some of the transgenic mice are deficient in their responses to intracellular pathogens. Figure 5 shows survival curves for wild-type, Tg24, and Tg33 mice after L. monocytogenes inoculation. The LD₅₀ for both transgenic lines (~5 × 10⁶) is more than 10-fold lower than that for wild-type mice. Tg25 mice survived L. monocytogenes challenge as well as wild-type mice.

To test whether the increased susceptibility of Tg24 mice could be generalized to other intracellular pathogens, we challenged them with M. tuberculosis inoculation, then enumerated bacteria in several organs 40 days after injection. Table II shows that the number of CFU in the organs of transgenic mice was 0.5 to 1.5 logs greater than that in wild-type mice.

**Discussion**

To correlate the chemoattractant properties of MCP-1 in vitro with in vivo observations and to determine whether MCP-1 might have other activities, we constructed the transgenic mice described in this report. Surprisingly, despite the expression of high levels of biologically active protein, organs that expressed MCP-1 constitutively were not ravaged by monocytic infiltration. One explanation is that the MMTV-LTR drove transgene expression at such high levels that local production of MCP-1 was more than 10-fold lower than that for wild-type mice. Tg25 mice survived L. monocytogenes challenge as well as wild-type mice.

**Table I. Serum concentrations of MCP-1**

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<thead>
<tr>
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<th>MCP-1 (ng/ml)</th>
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<tbody>
<tr>
<td>Wild-type (2)</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Tg24 (3)</td>
<td>13.1 ± 4.9</td>
</tr>
<tr>
<td>Tg25 (2)</td>
<td>4.1 ± 4.7</td>
</tr>
<tr>
<td>Tg33 (3)</td>
<td>25.6 ± 1.2</td>
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* Serum concentration of MCP-1 measured by ELISA, as described in Materials and Methods.

* Concentration ± standard deviation.

* Number of mice tested in each group.

The same as those that elicit a maximal monocyte chemotaxis response in vitro, and the level in Tg25 mice is near the EC₅₀ (15).

**Response to intracellular pathogens**

Transgenic mice (except for the small proportion of Tg33 mice) are healthy and long-lived in pathogen-free conditions, and they are capable of mounting histologically normal foreign body responses to material in surgical wounds (data not shown). This suggests that their monocytes are not completely inactivated. However, some of the transgenic mice are deficient in their responses...
levels of MCP-1 which were at the EC₅₀ for MCP-1-mediated monocyte chemotaxis in vitro. This amount may have been sufficient to prevent monocyte infiltration, but not high enough to produce the phenotype of increased *L. monocytogenes* susceptibility.

We have not determined whether the systemic inactivation of monocytes in some lines is due to receptor desensitization or simply to the physical loss of a chemoattractant gradient. In the former case, high levels of MCP-1 produced in specific organs may place monocytes on the descending limb of the bell-shaped chemotactant response curve. One way to distinguish between these possibilities would be to test transgenic monocytes for their ability to respond chemotactically to MCP-1 in vitro. Because of the small number of circulating monocytes in the mouse, we have been unable to perform this experiment. However, regardless of the mechanism, our results are analogous to those of Simonet et al., who suggested that the transgenic model is one of monocyte desensitization to MCP-1, a more reliable model of unresponsiveness to MCP-1 will be MCP-1⁻/⁻ mice created by targeted gene disruption. However, the phenotype of MMTV-MCP-1 transgenic mice may be distinct from the MCP-1⁻/⁻ model to whatever extent the former mice may have desensitized their MCP-1 receptors. In this case, the transgenic overexpressors will be desensitized to MCP-3 and any other as yet uncharacterized chemokines that activate the MCP-1 receptor. Targeted disruption of MCP-1 should generate a more limited defect, which may have a different phenotype from that of the mice described in this report.

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### References

