Abnormal macrophages in MRL-lpr mice are implicated in the pathogenesis of autoimmune disease. These mice die of lupus nephritis by 5 to 6 months of age. This study reports that MRL-lpr mice have an increased level of circulating macrophage colony-stimulating factor (M-CSF) detectable as early as 1 week of age. Macrophage colony-stimulating factor decreased between 2 and 4 months and then steadily increased beginning at 4 months of age. In contrast, M-CSF was not detected in sera from congeneric MRL-++ mice, normal C3H/FeJ mice, two other mouse strains with the lpr gene (B6-lpr and C3H-lpr), or another lupus model, the NZB/W mouse. These observations indicate that the lpr gene alone is not responsible for inducing this growth factor, and elevated M-CSF is not required for all forms of murine lupus. The entire source of serum M-CSF is not clear. The unique T cells regulated by the lpr gene are not responsible for the increased serum M-CSF levels, as no M-CSFs could be detected in supernatants from cultured lymph nodes from MRL-lpr mice, and the steady-state levels of M-CSF mRNA in lymph nodes and spleens in MRL-lpr, C3H-lpr mice and in their respective congenic strains were similar. The steady-state M-CSF mRNA transcripts in liver, lung, and bone marrow in MRL-lpr, MRL-++, and C3H/FeJ mice were also similar. Macrophage colony-stimulating factor transcripts were clearly elevated in the kidneys of MRL-lpr mice, suggesting a renal source of circulating M-CSF. The increase of M-CSF might be responsible for the increased numbers and enhanced functions of macrophages, which in turn cause tissue destruction in MRL-lpr mice (Am J Pathol 1991, 139:255–261)

Most studies of autoimmunity in MRL/MpJ-lpr/lpr (MRL-lpr) lupus mice have concentrated on the prominent and unusual T cells that are regulated by the lpr gene. Several descriptions of macrophage alterations, however, suggest that they play a critical role in the pathogenesis of autoimmune disease. We and others have previously reported increased numbers of peritoneal macrophages from MRL-lpr mice as compared with normal mice. These macrophages are more activated than those from normal mice, as they express unusually high levels of l a antigens, produce more hydrogen peroxide in response to phorbol myristate acetate stimulation, and have increased antibody-dependent cellular cytotoxicity against sheep erythrocytes. Furthermore MRL-lpr macrophages produce higher levels of the proinflammatory 5-lipoxygenase metabolite, leukotriene C₄, spontaneously and in response to zymosan, than macrophages from normal mice. Macrophages are rarely present in normal renal glomeruli, but can be isolated in MRL-lpr glomeruli before proteinuria and are even more prominent as the glomerulonephritis progresses. We have reported an elevation of interleukin-1 (IL-1) and tumor necrosis factor (TNF), cytokines produced predominantly by macrophages, in MRL-lpr kidneys with lupus nephritis. In this study, we investigated whether abnormal colony-stimulating factor (CSF) expression might be responsible for the altered numbers and activities of the macrophages in MRL-lpr mice. We detected increased levels of macrophage CSF (M-CSF) in sera from MRL-lpr mice, beginning as early as 1 week of age. In contrast, congeneric MRL-++ mice, C3H/FeJ mice, two other strains bearing the lpr gene (B6-lpr, C3H-lpr), and NZB ×...
NZW F₁ hybrid (NZB/W) mice, another autoimmune lupus model, did not have detectable circulating CSF at any age. The kidney, but not the liver, lung, bone marrow, or T cells regulated by the Ipr gene, is a source of increased M-CSF in MRL-Ipr mice at least by 4 months of age. Increased M-CSF may be responsible for macrophage proliferation and heightened function, which could in turn cause autoimmune tissue destruction.

Materials and Methods

Mice, Sera, and Proteinuria

MRL-Ipr, MRL/MpJ-/+ (MRL-+ +), C3H/FeJ, and NZB/W female mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. C3H/HeN mice were obtained from Charles River Laboratories (Wilmington, MA). Sera from B6-Ipr/Ipr (B6-Ipr) and C3H-Ipr/Ipr (C3H-Ipr) MRL-Ipr female and male mice were provided by Dr. John Roths, The Jackson Laboratory. Mice were bled by cardiac puncture or retro-orbitally after suitable anesthesia. The blood was allowed to clot at 4°C, centrifuged, and the serum stored frozen until use. Colony-stimulating factor-positive control serum was obtained from C3H/HeN male mice injected with 10 μg lipopolysaccharide (LPS) subcutaneously and bled 6 hours later. Previous data from our laboratory indicate that peak induction of M-CSF after LPS occurs between 6 and 8 hours and is absent by 18 to 24 hours. Mice were considered to have pathologic proteinuria when their protein levels were greater than 1.2 mg/24 hours by the trichloroacetic acid method.²

Colony-stimulating Assays

Bone marrow cells were obtained from the tibias of 20-g male C3H/HeN mice and cultured in duplicate in soft agar.¹ Briefly, 10⁵ bone marrow cells were added to 30 μl test serum and plated in 1 ml 0.3% Noble agar (Difco, Detroit, MI) in McCoy's 5A medium (Gibco, Grand Island, NY) supplemented with 15% fetal calf serum (HyClone, Logan, UT), 50 μmol/l (micromolar) 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), 10 U/ml penicillin, and 10 μg/ml streptomycin (Gibco) in 35-mm tissue culture plates (Costar, Cambridge, MA). Cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere and the colonies counted on day 9 or 10 with a dissecting microscope. Results of colony-stimulating activity (CSA) are reported as colony-forming units (CFU)/10⁶ bone marrow cells. This assay has a limit of detection of 50 units of M-CSF, based on determination of CFU using murine r-M-CSF. For inhibition studies, 30 μl of test sera was preincubated with 0.1 ml of various dilutions of either polyclonal rabbit anti-mouse L-cell–derived M-CSF (provided by Dr. R. Shadduck, Montefiore Hospital, Pittsburgh, PA) or normal rabbit serum for 30 minutes at room temperature before assay. The specificity of M-CSF in serum was tested by preincubation with M-CSF–containing L-cell supernatant, recombinant murine granulocyte macrophage CSF (Genzyme Corp., Cambridge, MA), and murine recombinant interleukin-3 (Biogen, Cambridge, MA).

Northern Blot Analysis

RNA was purified from freshly dissected tissues of normal and lupus mice using either cesium chloride gradient centrifugation¹⁰ or the acid-guanidinium-phenol-chloroform method.¹¹ RNA was glyoxylated and electrophoresed on 1.5% agarose gels, blotted onto Genescreen (New England Nuclear, Boston, MA), baked, and hybridized as previously described.⁶ The 2-kb M-CSF cDNA probe was provided by Dr. Steven Clarke (Genetics Institute, Cambridge, MA) and 32P-labeled using random hexamer primers (Pharmacia, Gaithersburg, MD).

Results

Elevated Serum CSF in MRL-Ipr Mice

Using the soft agar colony-stimulating assay, we detected elevated serum levels of CSF in MRL-Ipr lupus mice. At 1 week of age, CSF was elevated (47.1 ± 8.1 CFU) (Table 1). Circulating levels decreased after 1

Table 1. Elevated Levels of Colony-stimulating Activity in Sera from MRL-Ipr Mice at Various Ages

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age</th>
<th>Colony-forming units ± SE</th>
<th>n*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRL-Ipr‡</td>
<td>1 wks</td>
<td>47.1 ± 8.1</td>
<td>7</td>
</tr>
<tr>
<td>MRL-Ipr‡</td>
<td>5 wks</td>
<td>29.0 ± 3.5</td>
<td>3</td>
</tr>
<tr>
<td>MRL-Ipr‡</td>
<td>2 mos.</td>
<td>13.3 ± 4.3</td>
<td>3</td>
</tr>
<tr>
<td>MRL-Ipr‡</td>
<td>4 mos.</td>
<td>63.5 ± 0</td>
<td>2</td>
</tr>
<tr>
<td>MRL-Ipr‡</td>
<td>5–10 mos.</td>
<td>108.7 ± 6.3</td>
<td>2</td>
</tr>
<tr>
<td>MRL-+ +</td>
<td>1–9 mos.</td>
<td>1.1 ± 0.6</td>
<td>8</td>
</tr>
<tr>
<td>C3H/FeJ</td>
<td>3–5 wks.</td>
<td>0.8 ± 0.2</td>
<td>24</td>
</tr>
<tr>
<td>C3H/FeJ</td>
<td>2–10 mos.</td>
<td>0 ± 0.3</td>
<td>3</td>
</tr>
<tr>
<td>C3H/HeN + LPS§</td>
<td>2 mos.</td>
<td>133 ± 1</td>
<td>1</td>
</tr>
</tbody>
</table>

10⁴ C3H/HeN bone marrow cells were cultured in duplicates in soft agar with 30 μl of serum from various mouse strains and the colonies counted after 9 or 10 days. Data are reported as colony-forming units (X ± SE).

* n, number of mice tested.

† Additional analyses: 10 mice 1 to 4 months of age = 10.5 ± 3.1 CFU; 15 mice 4 to 6 months of age = 44.9 ± 5.0 CFU (P < 0.0001 by the Mann–Whitney U test).

§ Control obtained from pooling sera from mice injected with 10 μg of LPS given 6 hours before bleeding.
week, but were still readily detectable. Macrophage colony-stimulating factor levels were highest in MRL-lpr mice 4 months of age and older (Tables 1, 2). In fact, as determined in additional assays of 25 MRL-lpr mice ranging from 1 to 6 months of age, the highest levels of M-CSF were always in MRL-lpr mice 4 months of age and older. In mice 1 to 4 months of age, M-CSF = 10.5 ± 3.1 CFU and in mice 4 to 6 months of age, M-CSF = 44.9 ± 5.0 CFU (P < 0.0001). In contrast, C3H/FeJ mice at 3 to 5 weeks and 2 to 10 months of age, and congenic MRL-++ mice at 1 to 9 months of age expressed little if any serum CSA (0 ± 0.8 ± 0.2, and 1.1 ± 0.6 CFU, respectively) (Table 1).

Increased Serum CSA Is Increased More in Female MRL-lpr Mice

Because the 50% mortality rate occurs 1 month earlier in female than in male MRL-lpr mice, we compared sera of age-matched female and male mice. Colony-stimulating activity was higher in females than in age-matched male MRL-lpr mice (Table 2). In MRL-lpr mice at 1 month of age, the mean value was 25.0 ± 8.0 CFU in females and 10.5 ± 6.7 CFU in males. By 4 months of age, CFUs doubled in each sex, while maintaining the differential gender effect.

Increased Serum M-CSF Is Not Regulated by the lpr Gene

Because MRL-++ mice did not have circulating CSA, we investigated whether the enhanced CSA was linked to the lpr gene by analyzing sera from B6-lpr and C3H-lpr mice. The expression of elevated CSA in the MRL-lpr mouse strain is clearly not regulated by the lpr gene alone, as sera from these mice did not exhibit increased levels of circulating CSA (Table 2). We also investigated whether another autoimmune lupus mouse, the NZB/W hybrid, has enhanced serum CSA levels. As can be seen from Table 3, these mice did not exhibit elevated serum CSA levels (1.4 ± 0.5 CFU), whereas control sera from a

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age (months)</th>
<th>Colony-forming units</th>
<th>n²</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6-lpr†</td>
<td>3-4</td>
<td>2.6 ± 0.4</td>
<td>4</td>
</tr>
<tr>
<td>C3H-lpr†</td>
<td>2</td>
<td>2.0 ± 0.7</td>
<td>4</td>
</tr>
<tr>
<td>MRL-lpr</td>
<td>5</td>
<td>73.0</td>
<td>1</td>
</tr>
<tr>
<td>NZB/W</td>
<td>2-7</td>
<td>1.4 ± 0.5</td>
<td>10</td>
</tr>
<tr>
<td>C3H/HeN + LPS‡</td>
<td>2</td>
<td>108.0</td>
<td>1</td>
</tr>
<tr>
<td>B6-++†</td>
<td>3-4</td>
<td>5.0 ± 3.0</td>
<td>3</td>
</tr>
<tr>
<td>MRL-lpr</td>
<td>3</td>
<td>53.0 ± 11.0</td>
<td>3</td>
</tr>
<tr>
<td>C3H/HeN + LPS‡</td>
<td>2</td>
<td>91.0</td>
<td>1</td>
</tr>
</tbody>
</table>

* See Table 1 for explanations.
† Sera provided by Dr. John Roths, the Jackson Laboratory, Bar Harbor, Maine.
‡ Control obtained from pooling sera from mice injected with 10 µg of LPS 6 hours before bleeding.

Table 3. B6-lpr, C3H-lpr, NZB/W and B6-++ mice do not have detectable serum colony-stimulating activity

5-month-old MRL-lpr and LPS-injected C3H/HeN mice gave typical colony counts (73 and 108 CFU, respectively). In addition, because B6-lpr and C3H-lpr sera were supplied by Dr. John Roths at the Jackson Laboratory, we measured serum CSA in MRL-lpr and B6-++ from his colony as well, to eliminate the effect of mouse colony variability on CSA. MRL-lpr mouse sera provided by John Roths had similar levels of CSA as compared with sera from our colony. In addition, the B6-++ mice had nearly undetectable circulating CSA (Table 3, Experiment 2).

M-CSF Is Responsible for Increasing Serum CSA

Detectable CSA in MRL-lpr serum is from circulating M-CSF, alone. MRL-lpr CSA was inhibited by 30-minute pretreatment of MRL-lpr serum with high dilutions of specific polyclonal rabbit antimurine M-CSF; whereas normal rabbit serum had little effect on CFUs (Table 4). In another experiment, CSA in sera from four different MRL-lpr mice was completely inhibited by a 1:320 dilution of anti-M-CSF serum, whereas normal rabbit serum had no effect (data not shown). Finally anti-M-CSF was specific, as it neutralized L cell M-CSF but not GM-CSF or IL-3 activity.

Source of Elevated M-CSF

To investigate the source of M-CSF responsible for increased circulating levels in MRL-lpr mice, we analyzed steady-state mRNA levels in lymph node, spleen, liver, lung, and bone marrow. Although the unique T cells regulated by the lpr gene are responsible for the dramatic lymph node and spleen enlargement,1 our data do not support the concept that the unique T cells, regulated by the lpr gene, are producing circulating M-CSF. Approxi-
Table 4. Preincubation with Anti-M-CSF Serum Inhibits MRL-Ipr Serum and L-cell Supernatant but not rGM-CSF or rlL-3 Induced Colony-stimulating Activity

<table>
<thead>
<tr>
<th>Anti-serum dilution</th>
<th>3-6 mos. MRL-Ipr serum</th>
<th>4 mos. MRL-Ipr serum</th>
<th>L-cell sup (25 µl)</th>
<th>rGM-CSF (50U)</th>
<th>rlL-3 (50U)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+NRS</td>
<td>+aM-CSF</td>
<td>+NRS</td>
<td>+aM-CSF</td>
<td>+NRS</td>
</tr>
<tr>
<td>1:20</td>
<td>52</td>
<td>0</td>
<td>39</td>
<td>0</td>
<td>63</td>
</tr>
<tr>
<td>1:80</td>
<td>58</td>
<td>0</td>
<td>45</td>
<td>0</td>
<td>63</td>
</tr>
<tr>
<td>1:320</td>
<td>45</td>
<td>0</td>
<td>44</td>
<td>0</td>
<td>69</td>
</tr>
<tr>
<td>1:1280</td>
<td>50</td>
<td>0</td>
<td>33</td>
<td>1</td>
<td>61</td>
</tr>
<tr>
<td>1:5120</td>
<td>55</td>
<td>4</td>
<td>33</td>
<td>6</td>
<td>60</td>
</tr>
<tr>
<td>0</td>
<td>48</td>
<td>40</td>
<td>67</td>
<td>67</td>
<td>86</td>
</tr>
</tbody>
</table>

* Pooled MRL-Ipr sera, L-cell supernatant, rGM-CSF, and rlL-3 were preincubated with normal rabbit serum (NRS) or rabbit anti-murine M-CSF serum (am-CSF) for 30 minutes before assay as described in Table 1.

mately equal steady-state levels of M-CSF mRNA were in lymph nodes samples taken from MRL-Ipr, MRL-++ C3H/FeJ, and C3H-Ipr strains (Figure 1). Because the lymph nodes in Ipr mice are 100-fold larger than those in their congenic strains, the total level of steady-state M-CSF mRNA is more abundant in mice with the Ipr gene. We were unable to detect increased circulating M-CSF in B6-Ipr and C3H-Ipr mice, however, and in another experiment we were unable to detect M-CSF in supernatants of enlarged lymph nodes from MRL-Ipr mice cultured for 24 hours. In addition, enlarged spleens of MRL-Ipr (3 months) and C3H/FeJ mice expressed equal levels of M-CSF mRNA, which declined in MRL-Ipr mice at 9 months of age (Figure 2). The liver and lung from MRL-Ipr, C3H/FeJ, and MRL-++ all expressed M-CSF transcripts; but there was only a modest increase of M-CSF transcripts in either of these tissues in the MRL-Ipr mice as compared with normal strains (Figure 3). By comparison, bone marrow cells from MRL-Ipr or C3H/FeJ mice expressed similar barely detectable levels of M-CSF transcripts. Thus the serum M-CSF levels do not appear to be contributed by liver, lung, or bone marrow cells.

The kidney may be responsible, at least in older MRL-Ipr mice, for increased circulating M-CSF levels. There was an age-related increase in M-CSF mRNA levels in MRL-Ipr cortical renal tissue (Figure 4). Although 2-month-old MRL-Ipr mice had modestly increased levels of M-CSF mRNA as compared with C3H/FeJ or MRL-++ mice 3 to 11 months of age, MRL-Ipr mice 4 to 5 months of age clearly had a marked increase in M-CSF transcripts. It is of interest that the circulating M-CSF levels were higher in MRL-Ipr mice at 4 months of age than in younger mice and that this level increased even higher in older mice with more severe renal damage.

Discussion

We now report an increase in circulating M-CSF as a possible cause of autoimmune disease in MRL-Ipr mice. An elevation of M-CSF in MRL-Ipr mice as early as at 1 week of age clearly precedes overt expression of many features of autoimmunity, including increased circulating IgG1 present at 1 month of age (Dr. C. L. Sidman, 1990, oral communication), lymphadenopathy at 2 to 3 months of age, mononuclear infiltrates and increased renal tubule expression of MHC class II beginning at 2 months of age, and the onset of glomerulonephritis at 3 to 4 months of age.12,13

This increase of M-CSF in the circulation is likely to increase the numbers of macrophages and alter macrophage function. Human M-CSF injected into normal mice for 4 days increased the numbers of bone marrow cells responsive to M-CSF and circulating mature monocytes.14 In addition, macrophages grown from bone marrow cells cultured in M-CSF in vitro are rendered more sensitive to interferon-γ (IFN-γ) stimulation of la expression. Therefore the elevated serum M-CSF may be responsible for both the increased numbers of macrophages and the higher proportion of la-positive macrophages reported in MRL-Ipr mice.2-4
One important result of increasing the numbers or activation of macrophages in MRL-lpr mice could be an increased secretion of cytokines in vascularized sites, which may be responsible for causing tissue damage in the MRL-lpr mice. In turn, M-CSF can stimulate the production of IFN-α, TNF, and CSA by human peripheral monocytes. We have demonstrated the presence of macrophages in the kidneys of MRL-lpr mice and an increase in TNF and IL-1 mRNA and product in the kidneys of mice with lupus nephritis. Other investigators report that monocyte-derived cells, Kupffer cells, isolated from MRL-lpr mice, secrete larger quantities of TNF and IL-1 than do these cells from control mice. Perhaps the elevated M-CSF in MRL-lpr mice is also responsible for these alterations in tissue cytokine production.

Because the B6-lpr, C3H-lpr, B6-+++, and MRL-++ mice do not have detectable serum CSA, it appears that the abnormal expression of M-CSF in MRL-lpr mice is not regulated by the lpr gene or the MRL background alone, but because of interactions of the gene on this background. Similarly the interaction of the lpr gene on the MRL background is required for rapidly progressive renal injury. Despite massive lymphoproliferation in several strains with the lpr gene (C3H, B6, Akr, and NZB), only MRL-lpr have increased numbers of activated peritoneal and glomerular macrophages and severe renal injury. Thus it is tempting to speculate that the increased M-CSF is responsible for increasing numbers of activated macrophages, which localize in the renal glomerulus and cause injury. It is of interest that circulating M-CSF is higher in age-matched female mice with more severe renal disease as compared with male MRL-lpr mice. Increased M-CSF, however, is not a prerequisite for renal disease in murine lupus. Another lupus strain, the NZB/W F1 female hybrid, develops a slower form of nephritis with the 50% mortality rate in females at 8 to 9 months of age, as compared with 5 to 6 months of age for female MRL-lpr mice. Circulating CSA was not detected in the NZB/W F1 female hybrid. In addition, others have reported normal numbers of macrophages in this hybrid. Clearly these data support previous evidence that, although there are some common features between these two models, the mechanisms of disease differ.

Our data presented here do not support the concept that a bone marrow defect is responsible for the abnormal M-CSF levels. In addition, preliminary experiments in which we transferred bone marrow cells from MRL-lpr mice into MRL-++ mice did not increase circulating M-CSF levels above serum levels of M-CSF in MRL-+ receiving MRL-++ bone marrow cells.

The kidney cortex was the only tissue with a detectable difference in M-CSF transcripts between MRL-lpr mice and normal or congenic mice. The age-related increase of M-CSF transcripts in renal tissue could be a result of an increased number of macrophages or mesangial cells in the kidney capable of generating M-CSF. Further studies are in progress to clarify the con-
A distribution of each of these cell types to the increase in renal and circulating M-CSF and to determine the source of the early elevation in circulating M-CSF.

Increased M-CSF may be important in the pathogenesis of autoimmunity. Abnormal hematopoiesis has been observed in various autoimmune mouse strains. The moth eaten mouse strain has an increase in the number of macrophages that appears to be caused by enhanced CSF production by the macrophages themselves. Furthermore sublethally irradiated MRL-Ipr, NZB, and female NZBW autoimmune mice generate higher numbers of splenic CFUs than C3H-Ipr or B6-Ipr mice, suggesting a greater hematopoietic potential in these autoimmune strains. Thus unregulated production of immune cells, such as macrophages, may be crucial to the expression of some forms of autoimmunity.

The persistent presence of high levels of M-CSF in the circulation of MRL-Ipr mice suggests a defect in M-CSF gene regulation. A defect of regulation may reside in the M-CSF gene itself, or in a regulatory sequence or binding factor that allows for increased stability of mRNA, product, or constitutive M-CSF synthesis. A defect in gene regulation of IL-1 expression in peritoneal macrophages from MRL-Ipr mice as young as 1 month of age has already been reported. In addition, a defect in C-myb gene regulation has been documented in these mice. Alternatively the defect may reside in the regulation of a stimulatory cytokine such as TNF, which is increased in MRL-Ipr mice and stimulates M-CSF production, in vivo. In addition, M-CSF could accumulate in the serum if it were not properly used or degraded. Further studies are focused on identifying the cells synthesizing and consuming M-CSF and determining if there is a regulatory defect in M-CSF or another cytokine responsible for increasing M-CSF in the circulation of MRL-Ipr mice.

Acknowledgments

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References

24. Mountz JD, Steinberg AD: Studies of c-myc gene regulation in MRL-Ipr/lpr mice. Identification of a 5'-c-myc nuclear protein binding site and high levels of binding factor in nuclear extracts of Ipr/lpr lymph node cells. J Immunol 1989, 142:328–335