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High Serum Level of Macrophage Colony-Stimulating Factor (M-CSF) in *lpr* and *gld* Autoimmune Mice

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Abstract: In order to study the pathogenesis of autoimmune disease, serum M-CSF levels were measured by soft agar method in autoimmune MRL/*lpr*, BXSB, C3H/*lpr*, C3H/*gld*, NZB/W F1 mice as well as control MRL/*n*, C3H/Hej, BALB/c mice. Circulating levels of M-CSF were higher in MRL/*lpr*, C3H/*lpr* and C3H/*gld* mice than control mice, indicating the contribution of the *lpr* and *gld* genes. The number of macrophage precursor cell in the spleen was also higher in MRL/*lpr*, mouse than MRL/*n* mouse. However, there were no differences in Ia-positive macrophages from the bone marrow or spleen between high M-CSF group and low M-CSF group.

Key words: M-CSF, Mouse, Autoimmune, *lpr, gld*

INTRODUCTION

MRL/*lpr* and *gld* mice are spontaneous murine models of human autoimmune diseases such as SLE (Systemic Lupus Erythematosus) or RA (Rheumatoid Arthritis)[1)]. Accumulated infiltration of macrophages in the lesions caused by glemerulonephritis, arthritis and arteritis have been reported as a characteristic histological features of these mice[2)]. So far various studies have demonstrated abnormal levels of cytokines in this model, including low production of IL-2 (Interleukin 2) and low reactivity to IL-2[3,4)], impaired production of IL-1[5)] (Interleukin 1) high levels of I-BCDF[6)] (*lpr*-B Cell Differentiation Factor) and high levels of serum IL-6[7)] (Interleukin 6). M-CSF is present in synovial fluid and culture supernatant of synovial tissue from patient with RA[8)], suggesting that macrophage activation is a primary event in RA. The present study has attempted to study the serum level of M-CSF in these autoimmune mice to explain these features.

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MATERIALS AND METHODS

CSF Assay;

Bone marrow cells were obtained from the femur of C57BL/6N male mice (Charles River Japan, Inc., Kanagawa, Japan) 8–12 weeks of age. Bone marrow cells (1×10^5) were plated in 1 m*l* McCoy's 5A medium (Gibco, Grand Island, NY, U.S.A.) containing 0.3 % soft agar (Difco, Detroit, MI, U.S.A.) and 20% heat inactivated FCS (Flow Laboratories, McLean, Va, U.S.A.) in 35 mm plastic petri dishes (Falcon, Nippon Becton Dickinson Co., Ltd., Fukushima, Japan) with 0.1 m*l* of sample serum, 100 u/m*l* penicillin and 50 μg/m*l* streptomycin. After the agar was solidified, the plate was incubated at 37°C in a humidified 7.5% of CO_2 atmosphere for 7 days. Over 50 cells were counted as a colony under an inverted microscope. One unit of CSF was

defined as one colony and CSF activity was expressed as Units/m*l*. Assay were performed in triplicate.

At least 500 cells per slide were differentially counted under a microscope after the gel was transferred to a glass plate and dried and stained with Papanicolaou's staining.

CSF subclass specific activity was examined by incubating samples for 2 hours at room temperature before assay with goat anti-murine M-CSF antiserum, kindly provided by Dr. E.R. Stanley, Albert Einstein College of Medicine, New York, normal goat serum or rabbit anti murine GM-CSF (Granulocyte-Macrophage Colony Stimulating Factor) antisera, kindly given by Dr. T. Sudo, Biomaterial Research Institute Co., Ltd., Yokohama, Japan, or normal rabbit serum.

Spontaneous CFU-C (Colony Forming Unit in Culture) of bone marrow or spleen cells from MRL/*lpr* or MRL/*n* mouse were similarly counted, using 1×10^5/m*l* of bone marrow cells or 1×10^6/m*l* of spleen cells.

The number of precursor cells of macrophage lineage (CFU-M) in the bone marrow, or spleen of MRL/*lpr* or MRL/*n* mice was examined by incubating 5×10^4/m*l* of bone marrow cells or 5×10^5/m*l* of spleen cells in a 0.3% agar plate with 2000 u. of rM-CSF (Genzyme Co. Cambridge, MA, U.S.A.) for 7 days in a humidified 7.5% CO_2 atmosphere.

Mice

MRL/*lpr* mice were originally purchased from the Jackson Laboratory, Bar Harbor, Maine, U.S.A., and maintained in our specific pathogen free animal facilities. MRL/*n* mice were originally a gift from Dr. C. Abe, Juntendo University, Tokyo, Japan and similarly maintained. C3H/*gld* and BXSB mice were originally gifts from Dr. A. Matsuzawa and Dr. T. Katagiri, Tokyo University, Tokyo, Japan and maintained as above.

Sera from NZB/W F1 mice of different ages and sexes were a girt from Prof. T. Shirai, Juntendo University, Tokyo, Japan. Sera from C3H/*lpr* mice were gifts from Dr. M. Nose, Tohoku University, Sendai, Japan. BALB/c and C3H/Hej mice were purchased from SLC, Hamamatsu, Japan.

The experiment was performed in accordance with the Guidelines for Animal Experiments, Yamanashi Medical University.

Serum

Blood from the retroorbital plexus of mice under ether anesthesia, was placed for 30 minutes at room temperature, then moved to 4°C for 2–3 hours. The serum was separated by centrifugation and stored at −20°C until use.

Flowcytometry

Spleen and bone marrow cells of MRL/*lpr*, MRL/*n* and C3H/Hej mice were stained either with anti-Mac 1 or Mac-2 or Mac-3 monoclonal antibody (Boehringer Mannheim Biochemica, Mannheim, Germany) or normal rat IgG together with biotin-anti-Ia^k monoclonal antibody (Meiji Institute of Health Science, Tokyo, Japan) or biotin conjugated normal mouse IgG followed by FITC-labeled anti-Rat IgG (TAGO, Inc., Gurlingame, CA, U.S.A.) and avidin PE (Becton Dickinson Japan Co., Tokyo, Japan). Two color flowcytometry was performed by using an EPICS Profile (Coulter Co., Hialeah, FI, U.S.A.).

Statistics

Statistical differences were calculated using unpaired Student's *t* test and p values below 0.05 were considered significant.

Results

Serum CSF Levels

As shown in Fig. 1, the average CSF levels in sera of male and female MRL/*lpr* mice were 2683±2100 (units/m*l*±SD) and 9483±1850 respectively. The latter was significantly higher than that in the male ($p<0.001$) at 7–9 months of age. Those of male and female MRL/*n* mice were 1031±1880 and 2343±1812 units/m*l*. Both male and female MRL/*lpr* mice had significantly higher CSF activity than the control congenic MRL/*n* mice ($p<0.0001$).

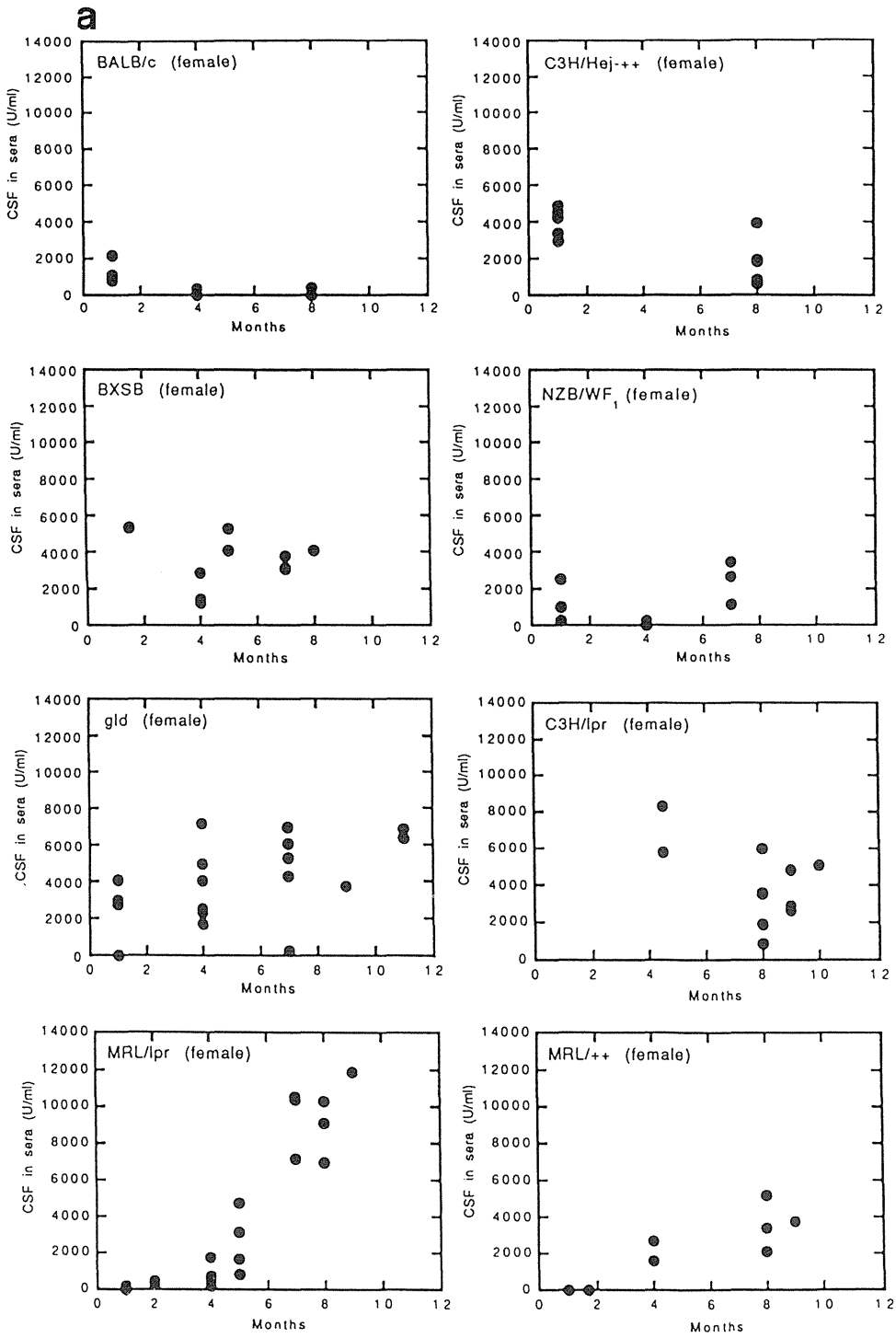
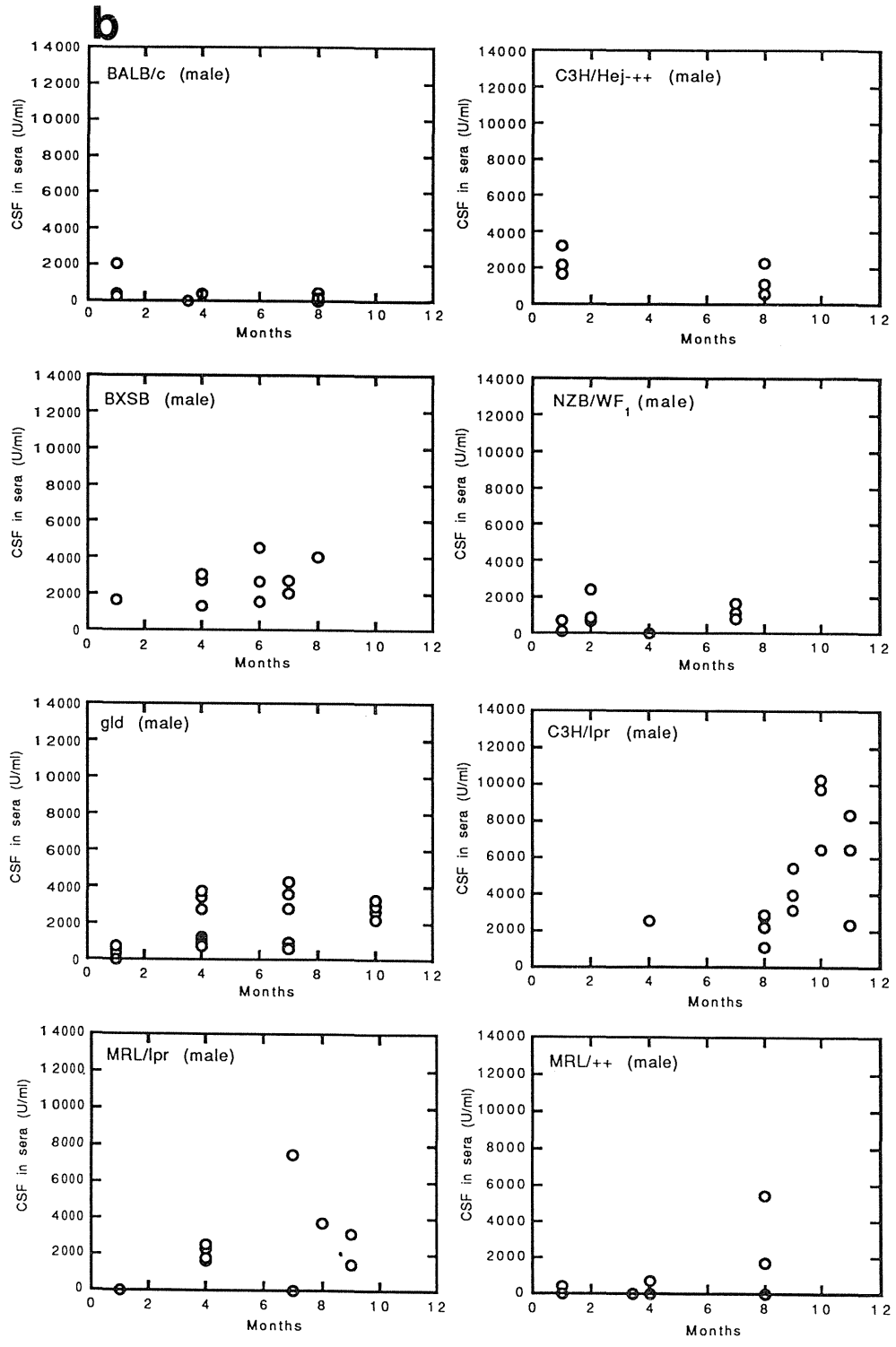


Fig. 1. CSF assay of sera from various strains of male and female mice at different ages.


b
BALB/c (male)
C3H/Hej-++ (male)
BXSB (male)
NZB/WF1 (male)
gld (male)
C3H/lpr (male)
MRL/lpr (male)
MRL/++ (male)
CSF in sera (U/ml)
Months

The levels in normal BALB/c mice were 419±213 in male and 587±217 in female respectively.

The male ($p<0.03$) and female ($p<0.0001$) MRL/*lpr* and the female ($p<0.03$) MRL/*n* mice had significantly higher levels of CSF than that of BALB/c mice. Autoimmune BXSB male and female mice had levels of 2638±1048 and 3607±1443 respectively. Both were significantly higher than those of BALB/c mice ($p<0.0001$, $p<0.0001$).

The female BXSB level was significantly less than that of MRL/*lpr* female ($p<0.004$).

Autoimmune male and female C3H/*gld* mice had levels of 1469±1152 and 2705±2555 respectively, whereas, those of congenic male and female C3H/Hej mice were 1916±803 and 2905±1534 respectively. Thus, *gld* mice had significantly higher CSF levels over 9 months than congenic males ($p<0.015$), female C3H/Hej ($p<0.011$), as well as BALB/c males over 7 months old ($p<0.0001$), females younger than 4 months ($p<0.019$) and females older than 7 months ($p<0.005$).

Table 1. Differential CSF assays of serum from various strains of mice were performed under a microscope after Papanicolau's staining. CSF was classified as macrophage (M) or granulocyte-macrophage (G/M) or granulocyte colonies G)

Strain	Sex	No	Femur /10^6BMC	/Femur ($\times 10^3$)	Spleen /10^6SC	/Spleen ($\times 10^3$)
MRL/*lpr*	M	1	1846	33.4	70.0	6.6
		2	1780	26.3	53.3	5.2
		3	1614	26.3	62.0	6.1
		4	1720	31.1	69.4	7.7
		5	2154	33.2	69.3	7.9
mean±SD				30.1±3.5		6.7±1.1
MRL/*n*	M	1	2306	29.1	72.0	4.4
		2	2180	41.4	3.4*	0.12*
		3	1900	34.6	35.4	2.4
		4	1634	27.5	39.4	2.8
		5	2554	39.8	72.7	4.3
mean±SD				34.5±6.2		3.5±1.0
MRL/*lpr*	F	1	1380	25.0	102.7	15.8
		2	1410	21.7	092.0	11.7
		3	1830	28.4	158.0	17.0
		4	1390	18.2	079.3	08.7
		5	1590	24.8	086.0	08.7
		6	1830	27.3	115.3	11.4
		7	1490	20.1	088.7	13.0
mean±SD				23.6±3.8		12.3±3.2
MRL/*n*	F	1	1110	14.2	72.0	6.1
		2	1400	25.9	32.7	2.7
		3	1258	20.2	52.4	4.4
mean±SD				20.1±8.3		4.4±2.4

BMC: Bone Marrow Cell, SC: Spleen Cell

The *gld* female had significantly lower CSF levels than MRL/*lpr* female mice ($p<0.004$). There were no significant differences between BXSB and *gld* mice of both sexes.

Autoimmune male and female NZB/W F1 mice had CSF levels of 756±230 and 1260±434 respectively. Thus, compared normal C3H/Hej, the levels of CSF were significantly lower in both sexes under 4 months old ($p<0.002$, $p<0.0001$). However, the male NZB/W F1 mice had higher levels of CSF than the normal C3H/Hej mouse($p<0.005$). However, there were no significant differences to normal BALB/c mice.

Thus, the MRL/*lpr* mice had the highest levels of CSF and BXSB and C3H/*gld* were the next. Autoimmune NZB/W F1 mice had almost same level as normal BALB/c mice. Generally, females had a higher titer than males. Pregnant mice were not included in this study, which were reported to have high titers of M-CSF[9,10].

Spontaneous CFU-C

The numbers of spontaneous CFU-C in spleen or bone marrow cells were all 0 in MRL/*lpr* and MRL/*n* mice, indicating that precursor cells in the bone marrow or spleen cannot differentiate *in vitro* without M-CSF.

The Number of Precursor Cells in The Bone Marrow or Spleen from MRL/lpr or MRL/n mice

As shown in Table 1, in the spleens of both males ($p<0.003$) and females ($p<0.0015$) the number of precursors were significantly higher in the MRL/*lpr* mice than in the MRL/*n* mice. Among the former mice, females had higher numbers than males ($p<0.004$). These phenomena corresponded with the circulating M-CSF level.

The lpr Gene

The *lpr* congenic C3H/*lpr* males and females over 8 months old had CSF levels of 4840±2980 and 4138±2123 respectively. Normal C3H/Hej males and females had levels of 1916±803 and 2318±1739 respectively, which were significantly lower (males, $p<0.01$ and females, $p<0.031$). Thus, the *lpr* gene may

Table 2. Goat anti-mouse M-CSF, or rabbit anti-mouse GM-CSF sera, or normal goat or rabbit sera were added to L-929 conditioned medium or mouse GM-CSF or MRL/*lpr* mouse serum, then colony formation was assayed

Mouse Strain	Age (month)	Sex	Colony type (%)		
			M	G/M	G
MRL/*lpr*	4	M	97	0	3
	7	M	74	12	14
	7	F	74	15	11
	8	M	80	16	14
	8	F	79	5	16
	9	M	76	16	8
	9	F	69	10	21
MRL/*n*	4	F	95	5	0
	8	M	100	0	0
	8	F	100	0	0
	9	F	100	0	0
C3H/Hej n	1	M	98	2	0
	1	F	97	3	0
	8	M	89	8	3
	8	F	94	0	6
C3H/*lpr*	4.5	F	80	13	7
	8	M	92	8	0
	8	F	75	22	3
	9	M	84	11	5
	9	F	87	11	2
	10	M	80	12	8
	10	F	76	15	9
	11	F	78	11	11
gld	4	F	88	10	2
	7	M	91	9	0
	7	F	94	5	1
	8	F	92	6	2
	10	M	93	2	5
	11	F	85	10	5
BXSB	1.5	F	69	12	19
	4	M	68	11	21
	4	F	82	11	7
	5	F	76	12	12
	6	M	77	10	13
	7	M	83	10	7
	7	F	83	6	11
	8	M	65	18	17
	8	F	67	17	16
NZB/WF1	1	M	82	6	12
	1	F	72	16	12

Table 3. Bone marrow spleen cells, 5×10^4 or 5×10^6/m*l* respectively were incubated in triplicate with rM-CSF 2000 u. in 0.3% soft agar for 7 days in a humidified 7.5% CO_2 atmosphere. The result is expressed as the mean±SD. excluded from the result

	L-929CM (0.5%)	mGM-CSF (100U)	MRL/*lpr* (2.5%)	medium
Anti-mM-CSF serum (×2,000 dilution)	8±2	145±4	22±1	4±1
Normal goat serum (×2,000 dilution)	172±15	149±6	170±15	3±1
Anti-mGM-CSF serum (×100 dilution)	126±3	6±2	116±13	5±1
Normal rabbit serum (×100 dilution)	155±11	146±15	144±12	5±1
anti-mM-CSF + anti-mGM-CSF serum	ND	ND	11±3	5±1
NGS + NRS	ND	ND	171±6	4±1

contribute to upregulate CSF activity, although the detailed mechanism remains unknown.

The Dominant CSF

As shown in Table 2, the majority of the colonies are of macrophages. To confirm the specificity, goat anti-mouse macrophages CSF antiserum was added to MRL/*lpr* mouse sera and the CSF level was assayed. As a control, normal goat serum was added at the same dilution.

As shown in Table 3, anti-macrophage antisera abrogated 87% of the CSF activity of MRL/*lpr* mouse serum compared with normal goat serum. Thus, most of the CSF derived from MRL/*lpr* mouse serum is considered to be M-CSF.

Ia-Positive Macrophages from Spleen and Bone Marrow in Various Strains of Mice

There were no significant differences in the percentage of Ia positive macrophages from spleen and bone marrow cells among MRL/*lpr*, MRL/*n*, C3H/ *gld*, and BALB/c mice, using monoclonal anti-Ia, Mac-1, 2, and 3 antibodies (data not shown).

Discussion

M-CSF promotes the growth and differentiation of bone marrow progenitor cells of mononuclear phagocytic lineage and can stimulate the proliferation of mature macrophages via specific cellular receptors[11]. Here, we report that the serum level of M-CSF in MRL/*lpr* is higher than that of MRL/*n* and also that C3H/*lpr* is higher than that of C3H/Hej mice, indicating the contribution of the *lpr* gene to elevated circulating M-CSF.

However, Yui *et al.*[12] reported that the *lpr* gene is not the cause of the elevated serum M-CSF level, based on the fact that there was no increased CSF activity in the sera of C3H/*lpr* and B6/*lpr* mice in comparison with control C3H/Hej and B6-+/+ mice, respectively. Their assay system was basically the same as ours, which was based on the colony formation of bone marrow cells on soft agar. The only difference is that they used bone marrow cells of male C3H/HeN mice whereas we used those of male C57BL/6N mice. Thus the reason for

this discrepancy remains unknown.

The *lpr* gene codes for an aberrant Fas antigen on the cell surface which allows cells to escape apoptosis[13]. So far, no data have suggested that escaped unusual T cells in swollen lymph nodes provide CSF. Thus, the relation between the aberrant Fas antigen and increased M-CSF remains unknown.

Here we also reported the elevated circulating M-CSF in the autoimmune C3H/HeJ-*gld*/*gld* mouse compared with C3H/Hej mice for the first time, indicating the contribution of the *gld* gene. The *gld* gene codes the aberrant Fas ligand and thus induces the same autoimmune symptoms as the *lpr* gene[14].

Here, in regards to M-CSF too, it is of notable that both genes express the same feature.

Recently the Fas ligand gene has been cloned[15] and the precise nature of the aberration in the *gld* gene has been clarified[16]. However, the finding that circulating M-CSF was not increased in other autoimmune mice such as NZB/W F1 and BXSB, suggests that elevated circulating M-CSF is not a common feature of autoimmune disease. Our report supports the data of Dang-Vu *et al.*[17] who found an increased number of peritoneal macrophages, increased function of ADCC and increased production of hydrogen peroxide in the MRL/*lpr* mouse in comparison with control C3H/HeN mice.

Expanded macrophage precursor populations in BXSB mice have been reported by Vieten *et al.*[18]. They claimed that this could be the cause of the peripheral monocytosis which was previously reported by Wofsy *et al.*[19] as a characteristic feature of this mouse. However, our data revealed only moderately elevated CSF in the serum of the BXSB mouse. Thus another mechanism may exist.

Along the same lines of evidence, Müller *et al.*[20] have reported that the numbers of both macrophages and macrophage precursors in the liver and spleen are increased in NZB/W F1 and MRL/*lpr* mice. In our study the M-CSF level in the serum was elevated more in MRL/*lpr* than in MRL/*n* mice and our data concurs with their finding of increased macrophage precursors. However, the level of serum M-CSF in the NZB/W F1 mouse was low, which is somewhat controversial. Thus, it is possible that in the NZB/W F1 mouse, there are more M-CSF receptors per cell, that they are more sensitive, or that other regulatory mechanisms exist. For example, Shieh *et al.*[21] reported that the M-CSF receptor is down modulated by TNF-α. Thus, the different level of TNF-α may influence the effect of M-CSF. Thus, more studies, especially of the M-CSF receptor in autoimmune mice, are required for further clarification.

About the source of M-CSF, Hora *et al.*[22] reported that IgG complexes could activate the synthesis of M-CSF in mouse mesangial cells. Since these autoimmune mice have high levels of circulating immune complexes in sera, the mesangial cells of these autoimmune mice may be stimulated by immune complexes to secrete high levels of M-CSF. The same group also reported that TNF-α also increased M-CSF production in mesangial cells[23].

In the MRL/*lpr* mouse, spontaneous production of TNF-α by Kupffer cells has been reported by Magilavy and Rothstein[24], Boswell *et al*[25], which supports this notion.

There are no differences between autoimmune and normal mice with regard to splenic and bone marrow derived Ia positive macrophages, as reported[26,27]. The reason why macrophage activation is limited to the peritoneum is unknown, and requires further study.

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