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Studies on Ly-1$^+$B Cells in Autoimmune-prone Strain of MRL/lpr Mice

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Ly-1 antigen is a surface antigen of murine T cells and is also expressed on the membrane of certain B cell population, especially the B cells in the peritoneal cavity of mice. Ly-1 antigen is the same molecule of CD5 antigen expressed on human T cells and a certain population of human B cells. It is suggested that Ly-1$^+$B cells are possibly involved in the pathogenesis of autoimmune diseases. In the present work, we analysed the profiles of the distribution of Ly-1$^+$B cells in the autoimmune-prone strain of MRL/lpr mice with different ages and normal mice (BALB/c and C57BL/6), because most of the studies for exploring the role of Ly-1$^+$B cells in the pathogenesis of autoimmune diseases have been carried out by using autoimmune-prone NZB-related mice. Two-parameter FACS analysis was used to investigate the percentage of Ly-1$^+$B cell subpopulation in spleens and peritoneal cavities of normal mice and autoimmune-prone MRL/lpr mice with different ages. It is shown that Ly-1$^+$B cell subpopulations in spleens and peritoneal cavities are present in increased number in MRL/lpr mice, suggesting an important role of Ly-1$^+$B cells in autoimmunity. In addition, older MRL/lpr mice showed higher number of Ly-1$^+$B cells as compared with those of young MRL/lpr mice.

Key Words: Ly-1$^+$B cells, Autoimmune disease, MRL/lpr, FACS analysis

INTRODUCTION

The body must establish self-tolerance mechanisms to distinguish between self and non-self antigenic determinants, so as to avoid autoreactivity. However, a number of diseases have been identified in which there is an autoimmunity due to the breakdown of self-tolerance mechanisms. Antibodies reacting to self-components (autoantibodies) have been shown to play a pathogenic role in some autoimmune diseases: Antibodies to DNA is a manifestation of the autoimmune disease of SLE (systemic lupus erythematosus) and may play an important role in pathogenesis of the disease in both human and murine SLE[1]. Studies on murine SLE provide a precious information and a model for elucidation of the pathogenesis of human SLE, since most of immunological abnormalities induced in human SLE appear to be operative in the murine SLE. NZB, NZB/W and MRL/lpr mice are the most commonly used as murine models for the investigation of human SLE.

Murine Ly-1$^+$B cells were first found in the peritoneal cavity. Ly-1 antigen that was originally demonstrated as a surface antigen of T cells is also expressed on the membrane of certain B cell population, especially the B cells in the peritoneal cavity. Ly-1 antigen is the same molecule of CD5 antigen expressed on human T cells and a certain population of human B cells. Ly-1$^+$B cells show a pattern of surface antigen expression distinct from conventional B cells[2]. The Mac1 (CD11b/CD18) antigen is co-expressed on peritoneal and pleural Ly-1$^+$B cells but not on conventional B cells. The anatomical localization of Ly-1$^+$B cells is also distinct from

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that of conventional B cells. The Ly-1$^+$B cells are the predominant B cells during fetal life. In adults, Ly-1$^+$B cells predominate in pleural and peritoneal cavities[3,4] and in the lamina propria of the gut as well[5], however, they account for only a few percentage of spleen B cells, and are rare in lymph nodes and peripheral blood[6].

It has been suggested by several groups that one of the important functional properties of Ly-1$^+$B cells is a possible involvement in the pathogenesis of autoimmune diseases[7~11]. Indeed, it has been shown that they spontaneously produce autoantibodies[3,11,12~15]. With autoimmune NZB mice, there were increased percentages of Ly-1$^+$B cells in both the spleen and peritoneal cavity[16] and that Ly-1$^+$B cell population from the mice secretes large amounts of IgM *in vitro*, accounting for virtually all of the spontaneous IgM secretion by spleen cells[6]. In humans, high-affinity antibodies to ssDNA were produced by CD5$^+$B cells in SLE patients[15], and higher percentages of CD5$^+$B cells were found in the blood of patients with rheumatoid arthritis[7,17], suggesting that CD5$^+$B cells might be involved in autoimmune diseases of human[13].

Fluorescence-activated cell sorter (FACS) studies have shown more convincing evidence of Ly-1$^+$B cells involvement in the pathogenesis of autoimmune diseases. Using two-color FACS analysis and sorting, it has been shown that the NZB B cells that spontaneously secrete IgM *in vitro* express Ly-1 antigen[6]. In addition, the number of Ly-1$^+$B cells was higher in NZB mice (5~10% in NZB spleens compared with 1~2% in BALB/c spleens)[6,18]. It has also been shown that Ly-1$^+$B cell subpopulation tended to be more active in NZB mice, since Ly-1$^+$B cells sorted from these animals spontaneously secreted much more IgM *in vitro* than Ly-1$^+$B cells sorted from BALB/c mice[6]. Hayakawa's group suggested that a high level of autoantibody production in NZB mice reflects defects in the regulation of Ly-1$^+$B cell percentage, activation, and differentiation rather than a general defect in the regulation of B-cell function[12].

The purpose of the present study was to study the profiles of distribution of Ly-1$^+$B cells in the autoimmune-prone strain of MRL/lpr mice with different ages and normal mice (BALB/c and C57BL/6). We used two-color FACS analysis to investigate the percentage of Ly-1$^+$B cell subpopulation in spleens and peritoneal cavities of these mice. We showed that Ly-1$^+$B cell population in spleens and peritoneal cavities are present at increased percentages in MRL/lpr mice, suggesting an important role of Ly-1$^+$B cells in autoimmunity. Moreover, older MRL/lpr mice (28~32 wks) showed higher ratio of Ly-1$^+$B cells as compared with those of young MRL/lpr mice (5~7 wks).

MATERIALS AND METHODS

Animals

BALB/c and C57BL/6 mice were purchased from Korea Experimental Animal Center, Seoul, and MRL/lpr from Korea Institute of Science and Technology, Taedok Science Town, Korea.

Reagents

FITC (Fluorescein)-conjugated goat anti-mouse IgM and PE (phycoerythrin)-conjugated rat anti-mouse Ly-1 monoclonal antibodies were purchased from Jackson ImmunoResearch Laboratories and Pharmingen, respectively. The reagents were used at the concentrations following manufacturer's instruction.

Preparation of lymphocytes

1) Spleen cells: After a spleen was removed from an animal, it was placed in 5 ml of cold DMEM (Dulbecco's Minimum Essential Medium) in a small petri dish. The spleen was gently disrupted by rubbing it on a metal mesh. Cells were transferred to a 15 ml conical tube in ice bucket and larger cell clumps were allowed to settle down for 5 min. The supernatant containing cells were passed through cotton-filled pasteur pipet and transferred to a clean 15 ml conical tube. This cell suspension was centrifuged at 300×g for 5 min, and the supernatant was aspirated from the pellet. The cell pellet was resuspended in Tris-buffered 0.14M NH_4Cl(pH 7.2) (0.1 ml packed cells/ml Tris-NH_4Cl), and the suspension was held at room temperature for 2 min. Fetal calf serum (FCS) was added to the sample and the sample was centrifuged at 300×g for 10 min. The cell pellet was washed twice with 5ml of cold media containing FCS before use. Viable cell number was counted after staining cells with Trypan blue.

2) Peritoneal cells: After a mouse was killed by cervical dislocation, about 5~10 ml of medium was carefully injected into peritoneal cavity. With the needle in place, the peritoneal cavity was massaged and then the fluid was carefully drawn

back into the syringe. After the fluid was passed through cotton-filled pasteur pipet and the same procedures as that for the spleen cells were followed.

Staining of lymphocytes

The concentration of cell suspension was adjusted to 2×10^7 cells/ml of staining solution (PBS containing 2% FCS and 0.1% sodium azide). Fifty μl of the cell suspension (total 1×10^6 cells) and 50 μl of goat anti-mouse IgM monoclonal antibody-FITC were added to Falcon 2058 tube and the content was mixed gently. Unstained cells were prepared by mixing 50 μl of 1×10^6 cells and 50 μl of staining solution for the control sample. The mixture was incubated for 30~45 min on ice, and 2ml of cold medium was added. The sample was centrifuged at $300\times g$ for 5 min at 4°C. After the supernatant was carefully aspirated from cell pellet, cells were resuspended in 50μl of staining solution and 50 μl of rat anti-mouse Ly-1 monoclonal antibody-PE for two-color staining and the same procedures as alone was followed. The cell pellet was resuspended in 0.5 ml of cold medium or fixed with 1% paraformaldehyde in PBS.

FACS analysis

Analyses were performed with Coherent INNOVA 300-laser FACS Vantage Flow Cytometer (Beckton Dickinson, California, U. S. A) equipped with logarithmic amplifiers to measure light scatter and the amount of pairs of fluorochrome-labeled monoclonal reagents bound to individual cells. During the analysis, a Macintosh Quadra 650 (Apple Computer, Cupertino, CA, U. S. A.) and CellQuest program were used to collect and store individual measurements on 100,000 cells for later analysis. Two-color staining data were presented as "dot plots" in which each dot represents one or more events (cells or particles). To generate statistics for a dot plot, quadrants were defined to divide dot plots into four sections. Location of quadrants was determined with controls including unstained lymphocytes and single stained lymphocytes with either FITC-antibody or PE-antibody. The statistics of each quadrant are calculated as percentage of the total events in a quadrant compared to the whole plot.

RESULTS

Each lymphocyte preparation from the spleens and peritoneal cavities of the C57BL/6 and BALB/c (8~16 wks), young MRL/lpr (5~7 wks) and old MRL/lpr (28~32 wks) mice were stained with FITC-conjugated anti-IgM antibody and PE conjugated anti-Ly-1 antibody in order to quantitate the Ly-1$^+$B cell population in each preparation. These populations were analyzed by FACS. In Fig. 1, unstained cells were shown in lower left quadrant, IgM$^+$/Ly-1$^+$B cells stained with both fluorochromes in upper right, IgM$^+$/Ly-1$^-$B cells stained with FITC-antibody in lower right and Ly-1$^+$/IgM$^-$ T cells stained with PE-antibody in upper left. A small subpopulation of splenic and peritoneal cells in normal mouse strains carried both IgM and Ly-1. Based on the FACS analysis, the percentages of Ly-1$^+$B cells were summarized in Table 1. As shown in Table 1, young BALB/c and C57BL/6 mice aged from 8 to 16 weeks have only 2.8% Ly-1$^+$ of total B cells in spleens. The peritoneal cavities of these mice show 3.3%

Table 1. Percentages of Ly-1$^+$ of total B cells

		Spleen		Peritoneal cavity	
		Average of total cell number	% of Ly-1$^+$B Cells	Average of total cell number	% of Ly-1$^+$B Cells
Normal	Young	2×10^7	2.8±2.1 (n=5)	ND*	3.3±2.7 (n=4)
MRL	Young	3.7×10^7	5.3±2.9 (n=3)	4.5×10^6	8.8±5.3 (n=3)
	Old	2.4×10^8	18.6±9.8 (n=4)	2.8×10^7	20.4±9.5 (n=3)

ND*: not determined

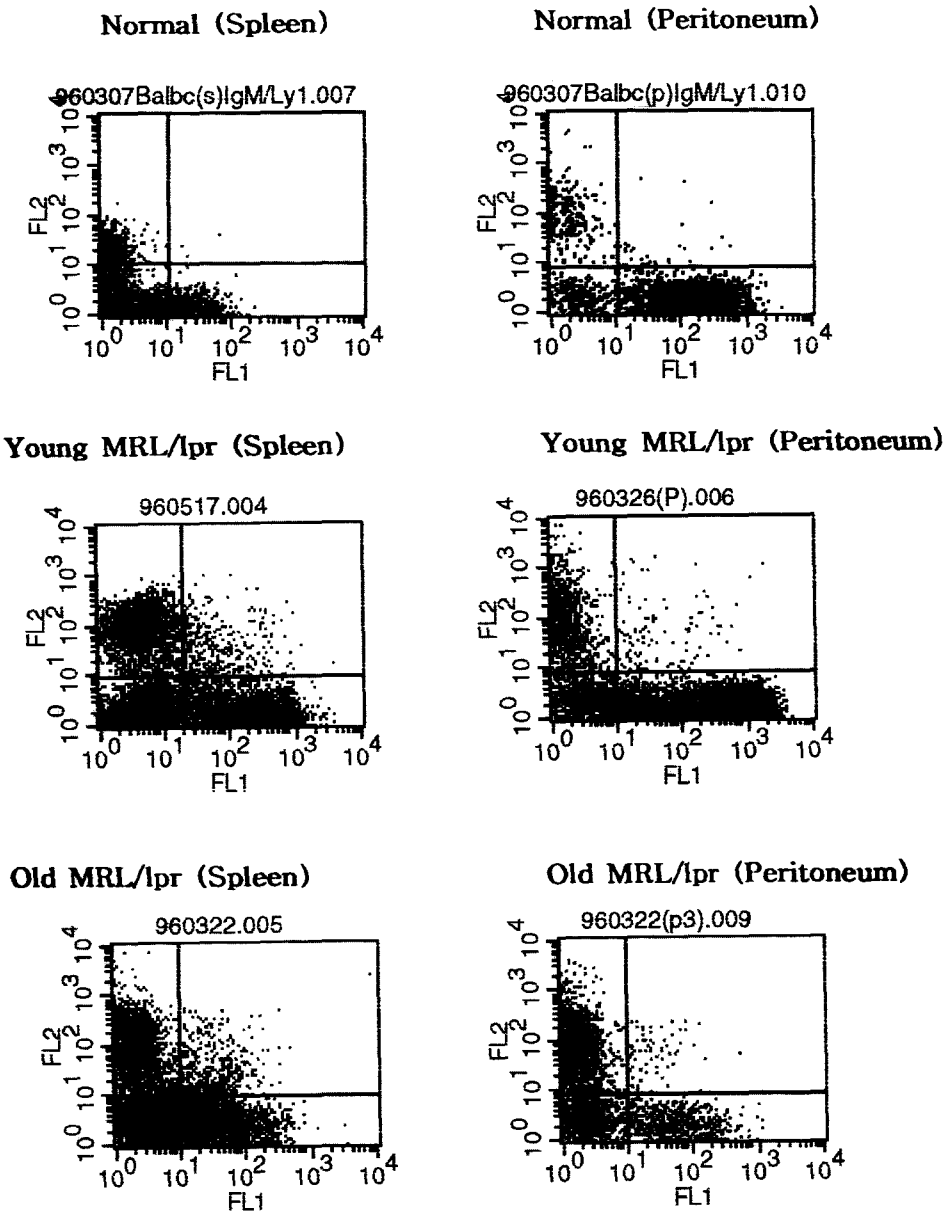

Fig. 1. Representative two-colour immunofluorescent staing profile of IgM+/Ly-1+ cells from normal and MRL/lpr mice: green fluorescence intensity (IgM-FITC) on X axis and red fluorescence intensity (Ly-1-PE) on Y axis(both in log scale). Based on control sample, the dot plots were divided into four quadrants: unstained cells (lower left); cells with both fluorochrome (upper right) or stained with either FITC-antibody (lower right) or PE-antibody (upper left). Numbers of lower right and upper right quadrants were added to calculate the number of total B cells.

Ly-1$^+$B cell of total B population. However, much larger subpopulation of splenic and peritoneal cavity B cells in MRL/lpr mice carried both IgM and Ly-1 (Fig. 1 and Table 1). Young MRL/lpr mice aged from 5 to 7 weeks have 5.3% Ly-1$^+$ of total B cells in the spleens. The peritoneal cavities of these MRL/lpr mice have even higher percentages (8.8%). The percentages increased remarkably (>20%) in both the spleens and peritoneal cavities of old MRL/lpr as compared with those of young MRL/lpr and normal mice. MRL/lpr mice are considered as old when they are older than 28 weeks which are equivalent to normal mice older than 24 months. At the time of sacrificing, MRL/lpr mice of 28 to 32 weeks-

old appeared to be very sick-looking and their spleens were abnormally large. As shown in Table 1, total cell number of old MRL/lpr was 5～10 times higher than those of young MRL/lpr and normal mice.

The peritoneal cells carried higher percentages of Ly-1$^+$B cells than the spleen cells. The autoimmune mice showed tendency of increased percentages of Ly-1$^+$B cell population compared to normal mice, although an individual mouse shows some variation. In old MRL/lpr, the percentages were almost same in both the spleens and peritoneal cavities. These results show that, as reported with NZB-related mice, the autoimmune MRL/lpr mice also have greater number of B cells carrying both IgM and Ly-1 than normal mouse strains.

DISCUSSION

Recently, a great deal of attention has been focused on a small subset of B lymphocytes that express Ly-1. It has been reported that about 5% of lymphocytes in the blood and lymphoid organs are Ly-1$^+$B cells and that they express a limited repertoire of V genes. Virtually all B cell-derived chronic lymphocytic leukemias are Ly-1$^+$. Moreover, Ly-1$^+$B cells spontaneously secrete IgM antibodies that often react with self antigens, and they may be expanded in the mice with autoimmune diseases. Experimental results suggest that Ly-1$^+$B cells are not developed in bone marrow, and a large number of these cells are found as a self-renewing population in the peritoneal cavity in mice. It is, therefore, likely that this small subset of B cells has unique properties in terms of ontogeny, function and role in induction of autoimmune diseases. Thus, it appears that the induction and pathogenesis of autoimmune diseases are closely associated with the presence of Ly-1$^+$B cells in mice. In order to elucidate this possibility, we carried out a study to determine the number and percentage of Ly-1$^+$B cells in autoimmune-prone strain of MRL/lpr mice with different ages.

MRL/lpr mice develope a spontaneous autoimmune disorder characterized by hypergammaglobulinemia, production of numerous autoantibodies, and very impressive lymphadenopathy, primarily due to the accumulation of enormous numbers of unusual B220$^+$, CD4$^-$ and CD8$^-$ T cells. The autoimmune disorder leads to premature death as early as 5～6 months of age under conventional housing. MRL/lpr strain of mice expresses abnormal Fas antigen on the lymphocytes due to the insertion of an early transposable element into an intron of the Fas gene, resulting in premature termination and aberrant splicing of the Fas antigen transcript. It has been known that Fas antigen expressed on the cell membrane induces apoptosis (programmed cell death) by interaction with Fas ligand or anti-Fas antibodies. The accumulation of lymphocytes on lymphoid organs in MRL/lpr is resulted from the defect of Fas antigen which may offer an explanation of lymphadenopathy and of the presence of auto-reactive lymphocytes.

In this study, we determined the numbers of Ly-1$^+$B cell from autoimmune-prone MRL/lpr mice with different ages and from normal mice by using quantitative two-color FACS analysis. As shown by our results, the percentages of Ly-1$^+$B cell in autoimmune-prone strain of old MRL/lpr are higher than those of young MRL/lpr and normal strains of mouse (C57BL/6 and BALB/c). Furthermore, the total number of Ly-1$^+$B lymphocytes from the spleens and peritoneal cavities of old MRL/lpr strain is greater than those of young MRL/lpr and normal mice. These observations are in good agreement with previous findings with NZB/W mice reported by many investigators: Ly-1$^+$B cells produce autoantibodies by polyclonal activation, which often react with self-antigens.

Thus, the spontaneous induction and pathogenesis of autoimmune disease in old MRL/lpr strain of mice are closely related with the generation of Ly-1$^+$B cells with higher percentage. The association of Ly-1$^+$B cells and autoimmune disease can be explained by the fact that Ly-1$^+$B cells produce a large amount of IgM antibodies that are able to react with self-antigens. Alternatively, since Ly-1$^+$B cells express restricted repertoire of antigen receptors, the Ly-1$^+$B cells can preferentially recognize self-antigens or bacterial antigens, which can crossreact with self-antigens. Therefore, the Ly-1$^+$B cells are activated by self-antigens or bacterial antigens to produce autoantibodies. To clearly define a role of Ly-1$^+$B cells in the pathogenesis of autoimmunity in MRL/lpr mice and SLE patients, however, further experiments are required.

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