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H-2^z Homozygous New Zealand Mice as a Model for B-Cell Chronic Lymphocytic Leukemia: Elevated *bcl*-2 Expression in CD5 B Cells at Premalignant and Malignant Stages

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In New Zealand mice, the major histocompatibility complex (MHC) controls the development of both autoimmune disease and B cell chronic lymphocytic leukemia (B-CLL). While H-2^d/H-2^z heterozygosity acts as one major predisposing genetic element for autoimmune disease, H-2^z/H-2^z homozygosity acts as an element for B-CLL. In the H-2^z/H-2^z homozygotes, there was an age-dependent increase in frequencies of CD5 B cells in the blood and spleen, and such CD5 B cells showed oligoclonal to monoclonal expansion, giving rise to B-CLL. B-CLL cells from these mice had surface phenotypes typical of CD5 B lineage cells, and expressed high levels of proto-oncogene *bcl*-2. Elevated *bcl*-2 expression was also observed in premalignant B cells in the aged mice, thereby suggesting that apoptosis-resistant, long-surviving CD5 B cells with a self-renewal capacity form the basis of malignant transformation. This model not only provides clues for analyzing multiple steps of genetic alterations involved in the generation of B-CLL, but also sheds light on the correlation between B-CLL and autoimmune disease.

Key words: B-CLL — MHC — New Zealand mouse — CD5 B cell — *bcl*-2 gene

Chronic lymphocytic leukemia (CLL) is a type of leukemia most often occurring among close relatives.[1,2] The frequent occurrence of immunological abnormalities, including autoimmune diseases, in patients with CLL and/or in their family members is another characteristic.[3–6] There is also a report that patients with CLL share HLA haplotypes with relatives with autoimmune diseases.[1] Since most cases of CLL seen in Caucasians are B cell-type CLL (B-CLL) with cell surface molecules of CD5 (Leu1), they are thought to originate from cells that belong to CD5 B cell lineage.[7–13] As opposed to the role of conventional B cells in acquired immunity, CD5 B cells mainly participate in the production of polyreactive natural antibodies and autoantibodies.[14] Thus, certain regulatory abnormalities in proliferation and differentiation of CD5 B cells are likely to be involved in both B-CLL and autoimmune disease.

In earlier studies on newly established H-2-congenic New Zealand mouse strains, NZB, NZW and (NZB×NZW)F1, we found that different, but related major histocompatibility complex (MHC) haplotypes predispose either to autoimmune disease or to B-CLL. While H-2^d/H-2^z heterozygosity acts as one genetic predisposing element for autoimmune disease, H-2^z/H-2^z homozygosity acts as one element for B-CLL.[15,16] All the New Zealand mice homozygous for H-2^z haplotype had abnormally high frequencies of splenic CD5 B cells, and such CD5 B cells showed an age-dependent oligoclonal to monoclonal expansion, giving rise to B-CLL.[17]

Chromosomal translocation related to the abnormal expression of *bcl*-2 and c-*myc* genes has been noted in human B-CLL.[18,19] Activation of proto-oncogenes such as c-*fgr* and *lck* was also proposed to be involved in the oncogenesis.[20] Therefore, the accumulation of multiple genetic alterations is likely to be involved in the malignant transformation of CD5 B cells. However, the process of this transformation is poorly understood, as there is a paucity in studies on premalignant B cells.

We now report that the H-2^z/H-2^z homozygous New Zealand mice show an age-associated increase of premalignant and malignant CD5 B cells in the blood, and thus can serve as an excellent spontaneous model of B-CLL and contribute to analyses of events occurring in the process of leukemogenesis, including the abnormal expression of proto-oncogenes.

MATERIALS AND METHODS

Mice NZB (H-2^d), NZW (H-2^z), (NZB×NZW)F1 (H-2^d/H-2^z) and C57BL/6 (H-2^b) mice were obtained from Shizuoka Animal Center (Shizuoka) and were maintained in our laboratory. H-2-congenic mouse strains (NZB.H-2^z and NZW.H-2^d) were established by selective backcrossing for 12 generations as described elsewhere.[21]

Flow cytometry Peripheral blood was obtained from periorbital sinuses and blood smears were stained with

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Giemsa solution. Total numbers of white blood cells (WBC) were measured using a Coulter counter (Coulter Co., Miami, FL). Spleen cell suspensions were obtained by gently dispersing the spleen tissue with a glass tissue grinder in RPMI 1640 with 5% fetal calf serum, followed by lysis of red blood cells with ammonium chloride. For flow cytometric analysis, aliquots of 5×10^5–10^6 cells in 20 μl of phosphate-buffered saline (pH 7.4) supplemented with 0.2% bovine serum albumin (BSA) and 0.05% NaN_3 were incubated with FITC-labeled antibodies and biotinylated antibodies, followed by phycoerythrin-avidin (Becton Dickinson, Mountain View, CA). All incubations were run for 30 min at 4°C. Monoclonal antibodies used were 331.12 (specific for mouse IgM),[22] CD5(Ly-1) (53-7.3),[23] RA3-6B2 (6B2),[24] CD45RA(Lp-2)[25] and CD11b(Mac-1).[26] The stained cells were examined using a FACStar (Becton Dickinson), equipped with the FITC/PE filter system. The lymphocyte population was gated by using forward vs. side scatter. Sorting of $CD5^+/IgM^+$ cells was done using the FACStar.

RT-PCR analysis Cytoplasmic RNA was extracted from the cells by the method of Favaloro *et al.*,[27] in the presence of the vanadylribonucleoside complex.[28] Mouse *bcl*-2 cDNA was synthesized using reverse transcriptase (RT) as follows: 750 ng of cytoplasmic RNA were hybridized with 1 pmol of the primer #1 (5′GCCATAT-AGTTCCACAAAGGCA) (sequence within exon 2 of the mouse *bcl*-2 gene),[29] in a total of 18 μl of the reaction mixture containing 55 m*M* Tris-HCl (pH 8.3), 83 m*M* KCl, 11 m*M* dithiothreitol, 3.3 m*M* $MgCl_2$ and 1.1 m*M* each of dGTP, dATP, dTTP and dCTP, at 65°C for 1 min, and then at 54°C for 5 min. The first strand of *bcl*-2 cDNA was synthesized by adding 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) and 10 units of human placental ribonuclease inhibitor (Takara Shuzo, Shiga), followed by incubation at 42°C for a further 15 min. Polymerase chain reaction (PCR)[30] was carried out by mixing 80 μl of PCR reaction mixture (12.5 m*M* Tris-HCl, pH 8.3, 62.5 m*M* KCl, 1.88 m*M* $MgCl_2$, and 0.0013% gelatin) containing 50 pmol of the primer #2 (5′ATGGCGCAAGCCGGGAGAACAGG-G) (sequence within exon 1 of the mouse *bcl*-2 gene[29]), 50 pmol of the primer #1 and 2 units of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) with thermal cycles of 93°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The total number of thermal cycles was 30. Ten μl of the PCR product was analyzed by 1% agarose gel electrophoresis, transferred to nylon membrane (Biodyne Nylon Membrane; Pall Biosupport Division, Glen Cove, NY) and probed with a 624 bp mouse *bcl*-2 cDNA. Mouse *bcl*-2 cDNA was obtained from spleen cells by PCR and subsequently cloned into a vector, pSKM13+. Southern blots thus obtained were quantitated using a BAS 2000 Bio-Imaging Analyzer (Fuji Photo Film Co., Tokyo).

Southern blot analysis Approximately 10 μg of genomic DNA, extracted from spleen cells, was digested with *Eco* RI, fractionated on 0.7% agarose gel, transferred to nitrocellulose filters, and probed with an Ig H-chain joining region (J_H) probe (PMJH), a 2.0-kb *Bam* HI/*Eco* RI fragment that includes J_H3 and J_H4 segments. The PMJH was radiolabeled by hexamer priming. Hybridizations were done in 6×SSPE/5×Denhardt's solution/0.5% SDS at 65°C (1×SSPE=0.18 *M* NaCl/10 m*M* sodium phosphate, pH 7.4/1 m*M* EDTA; 1×Denhardts's solution=0.02% BSA / 0.02% Ficoll / 0.02% polyvinylpyrrolidone).

RESULTS

Fig. 1 compares age-associated changes in the proportion of peripheral blood CD5 B cells in H-2-congenic NZB and NZW mice ($H\text{-}2^z/H\text{-}2^z$, $H\text{-}2^d/H\text{-}2^z$ and $H\text{-}2^d/H\text{-}2^d$). As compared with the mice either heterozygous for $H\text{-}2^d/H\text{-}2^z$ or homozygous for $H\text{-}2^d/H\text{-}2^d$, the $H\text{-}2^z/H\text{-}2^z$ homozygous mice showed a marked increase of CD5 B cells from 8 months of age onward.

Fig. 2 shows representative two-color flow cytometry profiles for sIgM and CD5 expressions of peripheral

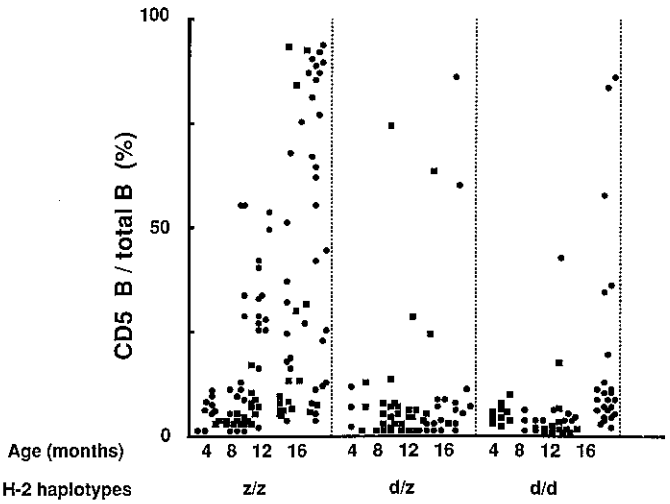

Fig. 1. Frequencies of CD5 B cells in peripheral blood B cells from H-2-congenic NZB (■) and NZW (●) mice ($H\text{-}2^z/H\text{-}2^z$, $H\text{-}2^d/H\text{-}2^z$ and $H\text{-}2^d/H\text{-}2^d$). Note that higher frequencies of CD5 B cells were observed for mice homozygous for $H\text{-}2^z$. Regression coefficients for the age-dependent increase of CD5 B cells are 4.1, 0.64 and 0.78 for $H\text{-}2^z/H\text{-}2^z$ homozygous, $H\text{-}2^d/H\text{-}2^z$ heterozygous and $H\text{-}2^d/H\text{-}2^d$ homozygous mice, respectively.

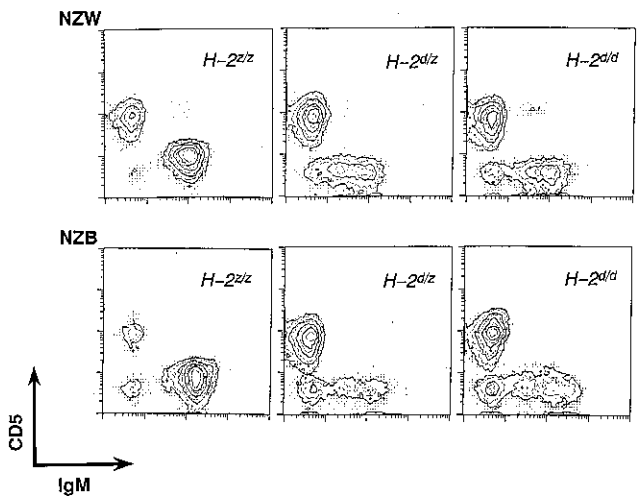

Fig. 2. Two-color flow cytometry profiles for sIgM and CD5 expressions. Peripheral blood lymphocytes from 12-month-old H-2-congenic NZW and NZB mice. Note that H-2^z/H-2^z homozygous mice showed remarkably high CD5 B cell frequencies, as compared to mice with other haplotypes (H-2^d/H-2^d or H-2^d/H-2^z). $CD5^+$ cells other than $CD5^+IgM^+$ cells are T cells with much higher CD5 expression.

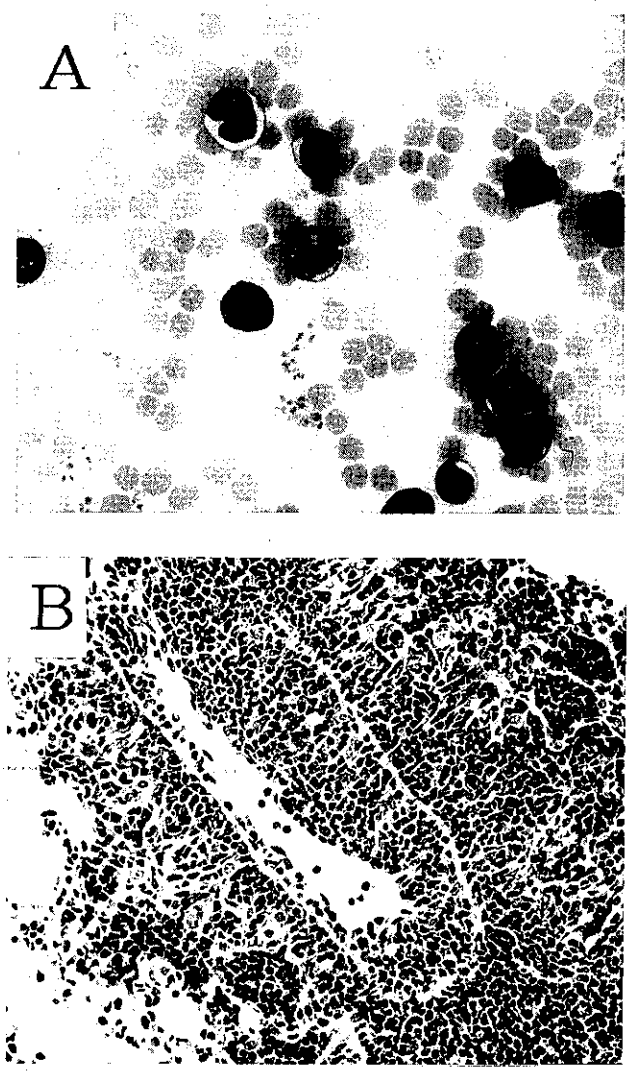

Fig. 3. A. Stained blood smear obtained from a 12-month-old NZW mouse with B-CLL. Leukemic cells have round nuclei and a high nuclear-to-cytoplasmic ratio. Owing to excessive fragility, neoplastic lymphocytes were often damaged, giving rise to several "smudge" cells. B. Histological picture of the lung obtained from a NZW mouse with B-CLL. Medium-sized leukemic cells have massively infiltrated in alveolar walls and subendothelial and perivascular spaces of a small vessel (HE).

blood lymphocytes obtained from premalignant 12-month-old H-2-congenic New Zealand mice. NZW and NZB mice with the homozygous H-2^z/H-2^z haplotype showed remarkably high CD5 B cell frequencies. These cells were also weakly positive for CD45R(6B2) and CD11b(Mac-1), a finding characteristic of CD5 B cells (data not shown). We obtained two transplantable B-CLL cell lines from these mice.

Stained blood smears obtained from NZW mice that showed expanded populations of CD5 B cells with homogeneous sIgM expression in the blood showed medium-sized B leukemic cells with round nuclei and a high nuclear-to-cytoplasmic ratio (Fig. 3A). Owing to excessive fragility, neoplastic lymphocytes were often damaged, giving rise to several "smudge" cells. Infiltrations of leukemic cells were observed in a wide variety of organs including spleen, lymph node, liver, kidney, salivary gland and lung (Fig. 3B), in keeping with the observations in human B-CLL.

To search for possible age-associated changes in the clonality of B cells, Southern hybridization analysis using a J_H probe was carried out with genomic DNA obtained from spleen cells of H-2^z/H-2^z hymozygous NZB mice of varying ages. Patterns of rearrangement of immunoglobulin DJ or VDJ gene segments changed from smear to oligoclonal and even to monoclonal bands, with increasing age of the animals (Fig. 4). These features were correlated well with the expansion of homogeneous $CD5^+$ $sIgM^+$ B cells on FACS profiles (data not shown).

Fig. 5 compares the levels of *bcl*-2 expression in transplantable B-CLL cell lines obtained from aged H-2^z/H-2^z homozygous NZW mice and spleen cells from young (2-month-old) and old (12-month-old) NZW mice. High levels of *bcl*-2 expression were observed for two transplantable B-CLL cell lines (lanes 1 and 2).

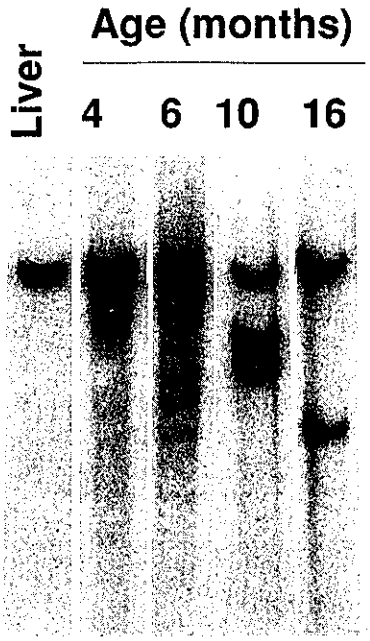

Fig. 4. Southern hybridization analysis using a J_H probe on genomic DNA obtained from spleen cells of H-2^z/H-2^z homozygous NZB mice at varying ages (4, 6, 10 and 16-month-old). Patterns of rearrangement of immunoglobulin DJ or VDJ gene segments changed from smear to oligoclonal and even to monoclonal bands with increasing age of the animals.

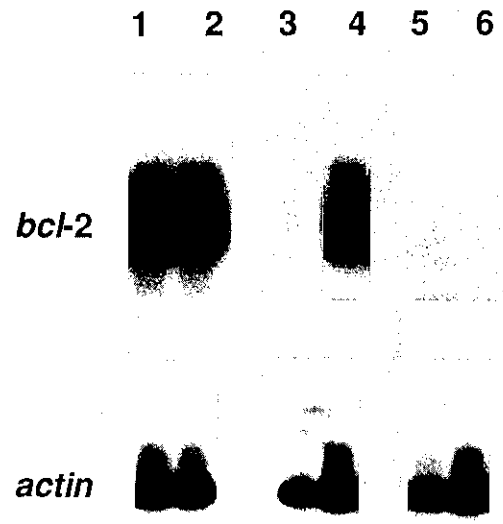

Fig. 5. Levels of *bcl*-2 expression in two transplantable B-CLL cell lines obtained from aged H-2^z/H-2^z homozygous NZW mice and spleen cells from young (2-month-old) and old (12-month-old) NZW mice. High levels of *bcl*-2 expression were observed for two transplantable B-CLL cell lines (lanes 1 and 2). There was an age-associated increase in the *bcl*-2 expression in spleen cells from NZW mice (lane 3; 2-month-old, lane 4; 12-month-old). No age-associated increase in the *bcl*-2 expression was observed for C57BL/6 mice (lane 5; 2 month-old, lane 6; 12-month-old). *bcl*-2 and actin mRNA were reverse-transcribed and subsequently amplified by polymerase chain reaction as described in "Materials and Methods."

There was also an age-associated increase in *bcl*-2 expression in spleen cells from NZW mice prior to the appearance of malignant cells in the blood (lanes 3 and 4). Such spleen cells from 12-month-old (NZW) mice contained CD5 B cells with oligoclonal rearrangements of J_H gene segments, by Southern blot analysis, but did not give rise to B-CLL when transplanted into young NZW mice. Thus, these cells were considered to be at a premalignant stage. Elevated levels of *bcl*-2 expression were also observed in sorted $CD5^+$ B cells in these mice at a premalignant stage (data not shown).

DISCUSSION

We found that New Zealand mice homozygous for H-2^z haplotype showed high frequencies of CD5 B cells in the blood in association with age-associated oligoclonal to monoclonal expansion of these cells. Such an expanded population of cells was transplantable and showed phenotypic features of $CD5^+$ B-CLL. Indeed, infiltrations of leukemic cells were observed in a wide variety of organs, in keeping with the observations in human B-CLL. Elevated *bcl*-2 expression was evident in CD5 B cells at both premalignant and malignant stages. Thus, New Zealand mice homozygous for H-2^z haplotype can serve as a suitable model to study the process of malignant transformation of CD5 B cells.

Murine CD5 B cells belong to a developmental lineage distinct from that of conventional B cells, have unique surface phenotypes, that is, $CD5^+$, dull $CD45R(6B2)^+$, bright $sIgM^+$, and $Mac\text{-}1^+$ (particularly CD5 B cells in the peritoneal cavity), are maintained by a self-renewal capacity and show a distinctly different localization pattern from conventional B cells.[14] Evidence is accumulating that CD5 B cells, both in mice and humans, only participate in natural immunity or in autoimmunity, as opposed to acquired immunity, and produce most of the IgM natural antibodies and autoantibodies, including those to DNA, thymocytes and erythrocytes.[14] Since New Zealand mouse strains have predispositions for autoimmune disease, such as systemic lupus erythematosus, autoimmune hemolytic anemia and Sjögren's syndrome,[31] and since CD5 B cells are likely to be responsi-

ble for such disease,[15,16] it is possible that self-reactive CD5 B cells with self-renewal capacity, which have escaped from clonal deletion mechanisms, are highly susceptible to genetic alterations related to leukemogenesis.

We observed high levels of proto-oncogene *bcl*-2 expressions in B-CLL cell lines obtained from H-2^z/H-2^z homozygous New Zealand mice. Proto-oncogene *bcl*-2 has been cloned as a gene activated by the translocation of t(14,18) in human follicular lymphoma.[32] Although details of the function of *bcl*-2 remain unclear, *bcl*-2 is thought to be responsible for the inhibition of programmed cell death (apoptosis), and B cells that overexpress *bcl*-2 exhibit enhanced cell survival.[33] While *bcl*-2 does not actively promote cell proliferation, it does provide a distinct survival signal to the cell.[33] Thus, *bcl*-2 may contribute to the leukemogenesis of B cells by allowing a clone to persist until other oncogenes become activated. This notion is consistent with our present finding that CD5 B cells at the premalignant stage already expressed high *bcl*-2. Further studies on involvement of other oncogenes in the leukemogenesis of B-CLL in the New Zealand mice are under way in our laboratory.

Human B-CLL cells have also been reported to express high levels of *bcl*-2[34] and in about 10%, the *bcl*-2 gene is translocated to one of the immunoglobulin loci.[35] Our preliminary studies using genomic Southern hybridization with several restriction enzymes showed no evidence of chromosomal translocation of *bcl*-2 gene (data not shown). To date, no murine B-CLL cell lines have been reported to have chromosomal translocation with respect to *bcl*-2.

The role of H-2^z/H-2^z homozygosity in the development of B-CLL remains unknown. A certain gene tightly linked to MHC is a possible candidate to be involved in the abnormality of CD5 B cells. The recessive effect of the gene is also reminiscent of a tumor suppression gene whose tumorigenic phenotype is recessive.[36] It is also tempting to speculate that difference in the polymorphism of MHC class II molecules determines the fate of CD5 B cells in New Zealand mice, i.e., either clonal expansion in H-2^z/H-2^z [17] or differentiation into autoantibody-producing cells in H-2^d/H-2^z mice[21] (although other genetic elements are also apparently involved in both autoimmune disease and B-CLL in these mice). The murine model of B-CLL we have described here will make feasible analysis of multiple steps of genetic alterations involved in the process of malignant transformation of CD5 B cells, as well as allowing us to evaluate putative correlations between B-CLL and autoimmune diseases.

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