The NZB Mouse as a Model for Chronic Lymphocytic Leukemia

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western world and the only leukemia for which a possible genetic component has been described. Analysis of this genetic component has been hindered by the fact that disease onset normally occurs after age 50. We report here the aged NZB mouse as an animal model for CLL. NZB mice have a genetically regulated, age-dependent onset of clonal, aneuploid cells which are IgM* and Ly1* (CD5* B-cells). Peripheral blood smears from old NZB mice show an increase in circulating lymphocytes and "smudged" or ruptured cells, often seen in human CLL. Electron microscopic examination of these cells shows them to be mature lymphocytes. Light microscopy of the spleen shows infiltration of small lymphocytes and is consistent with CLL pathology. These long-lived, CLL-like cells can be serially passed into recipient animals. This continued passage occasionally results in the development of a large cell lymphoma detectable in the spleen, lymph nodes, and liver. The histology of this lymphoma is quite distinct from that of the CLL-like cells, but the phenotype is that of an aneuploid CD5*, IgM* cell. This apparently represents a continued transformation of the CLL-like clone similar to the development of Richter's syndrome in human CLL. Therefore, the NZB mouse can be a valuable tool for the determination of the genetic basis of CLL ontogeny and the conversion of CLL into Richter's syndrome.

INTRODUCTION

CLL, the most common leukemia in the Western world, is a malignancy of the CD5+ B-cell (1-3). CLL typically presents as an accumulation of mature-appearing but biologically immature B-lymphocytes in a patient who is over 50 years of age (4). Diagnostic guidelines are based upon findings of lymphocytosis sustained for at least 4 weeks, B-cells with low-density surface immunoglobulin and the CD5 antigen, mature lymphocytes with no more than 55% stiypical or immature lymphoid cells, and bone marrow involvement of no more than 30% lymphocytes (5, 6). Despite its association with a host of immune abnormalities including B-cell dysfunction, increased absolute numbers of T-cells, hypogammaglobulinemia, and autoimmune responses to hematological antigens, CLL tends to run a somewhat indolent course over several years. Occasional cases, however, evolve into an aggressive, diffuse large cell lymphoma which can be either widespread or localized and is characterized by weight loss, localized mass, fever, and dysproteinemia. The presence of large cell lymphoma in CLL has been termed Richter's syndrome (7). The histology of Richter's syndrome is quite distinct from that of CLL. Growing evidence indicates that Richter's can result from either continued evolution of the CLL clone (8-13) or from the occurrence of a second, unrelated malignancy (14-17).

Old NZB mice develop a clonal expansion of CD5+ B-cells which have many similarities to the malignant cell in human CLL. By 1 year of age, the NZB displays clonal hyperplasia of a CD5+ B-cell which is long lived and slow growing and possesses specific extra chromosomes. These hyperdiploid CD5+ B-cells are long lived to the point of virtual immortality. We have previously shown that NZB hyperdiploid cells can be passaged by i.v. injection into unirradiated (NZB × DBA/2)F1 mice, where they will continue to proliferate (reviewed in Ref. 18). In the (NZB × DBA/2)F1 recipient of hyperdiploid cells, the expansion of these hyperdiploid CD5+ B-cells is associated with a corresponding hyperplasia of the recipient animal's own splenic T-cells and macrophages. The normal B-cells of the recipient animal are suppressed, as defined by a reduction in total splenic B-cell numbers and a decrease in serum IgM levels. In general, the passaged hyperdiploid cells are found mainly in the spleen, peripheral blood, and the peritoneal cavity of the recipient animal. However, following serial passage of a single clone through several recipients, we occasionally detect an aggressive lymphoma characterized by metastasis of the hyperdiploid clone into peripheral areas such as the lymph node and liver. This study examined the histology of the hyperdiploid CD5+ B-cell clone in both the old NZB, where they spontaneously develop, and in the passaged (NZB × DBA/2)F1 recipient, to see if they appear to be similar to CLL at the microscopic level. An (NZB × DBA/2)F1 recipient of cells from the secondary lymphoma was also examined to see if this murine lymphoma was analogous to the evolution of CLL to Richter's syndrome.

MATERIALS AND METHODS

Mice. NZB/N and DBA/2 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). (NZB × DBA/2)F1 mice were produced in the animal facilities at UMDNJ. Another substrain of NZB was also used in these studies. These mice were NZB/kmc Udì. These mice are very similar to NZB mice but develop clones of hyperdiploid CD5+ B-cells predominantly in the peritoneum rather than in the spleen, as is the case with NZB/N.

Spleen Cell Transfusions. All recipient animals are 2-month-old (NZB × DBA/2)F1 male mice which received 20 × 106 spleen cells by i.v. injection. The initial source of spleen cells was a female NZB mouse which possessed hyperdiploid CD5+ B-cells. Three to six months following transfer the mice were sacrificed, and their spleens were removed. A portion of the spleen was saved for histological studies, flow cytometric analysis, cytokine assays, and RNA and DNA extraction. Another portion of the spleen was used to obtain donor cells for a subsequent transfer into new F1 recipients. The primary NZB donor is referred to as I, the first recipient (an F1) is referred to as II, and so on (see Fig. 1).

Histological Analysis. For light microscopic examination, specimens were fixed in 10% formaldehyde solution, embedded in paraflin, and cut into 4-μm sections. The sections were stained with hematoxylin-eosin, Giemsa, and toluidine blue. Peripheral blood was collected in an EDTA tube, and smears were prepared and stained with Wright's stain. For electron microscopy, cells were embedded in LX-112 as described (19), with modifications to allow the examination of cell suspensions. Peritoneal wash cells were obtained by lavage with cold phosphate-buffered saline plus 1% bovine serum albumin (Sigma, St. Louis, MO), washed with phosphate-buffered saline, and pelleted. The cell pellet
Direct Lineage of the Richter's-like Clone

<table>
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<tr>
<th>Generation</th>
<th>Mouse_ID</th>
<th>Animal</th>
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<tbody>
<tr>
<td>I</td>
<td>8.1*</td>
<td>Year old NZB</td>
</tr>
<tr>
<td>II</td>
<td>13.2*</td>
<td>(NZB X DBA/2)F1 littermates</td>
</tr>
<tr>
<td>III</td>
<td>25.1* 28.1*</td>
<td>(NZB X DBA/2)F1 littermates</td>
</tr>
<tr>
<td>IV</td>
<td>32.1*</td>
<td>(NZB X DBA/2)F1 littermates</td>
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<tr>
<td>V</td>
<td>58.1*</td>
<td>(NZB X DBA/2)F1 littermates</td>
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<tr>
<td>VI</td>
<td>65.4 #</td>
<td>(NZB X DBA/2)F1 littermates</td>
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<tr>
<td>VII</td>
<td>72.1*</td>
<td>(NZB X DBA/2)F1 littermates</td>
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<tr>
<td>VIII</td>
<td>84.1**</td>
<td>(NZB X DBA/2)F1 littermates</td>
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* CLL-like; # Richter’s-like; * used in pathology

Fig. 1. Schematic view of the continued passage of a hyperdiploid CD5+ B-cell clone. Generation I is the 1-year-old NZB which originally developed the clone. Generations II-X are F1 mice which have been serially passaged with 20 x 10⁶ clone. Generation I is the 1-year-old NZB which originally developed the clone. The clone can be found in two or in (NZB x DBA/2)F1 mice. Young NZB mice do not display these clones; however, ~95% of NZB have detectable hyperdiploid clones by 1 year of age. The clone can be found in the peripheral blood, the spleen, and (occasionally) the peritoneal cavity or lymph node. Localization to these places identifies an animal as CLL-like. This is the case for old NZB and for some of the passaged F1 mice. Spread of the clone into the lymph nodes and the liver designates an animal as Richter’s-like. The Richter’s-like syndrome examined here arose in the second passaged F1 animal (Fig. 1, generation III). This line was first established in an old NZB (mouse 8.1; CLL-like) in 1986. Spleen cells from this NZB were passaged into an F1 animal (mouse 13.1), which also displayed a CLL-like pattern of distribution. The transformation to Richter’s occurred in the next generation. Of two F1 mice receiving transfused spleen cells from CLL-like mouse 13.1, one (mouse 28.1) retained the CLL-like characteristics and one (mouse 25.1) developed the Richter’s-like syndrome, with metastasis to the liver and lymph nodes. The line established in Richter’s-like mouse 25.1 has been continually passaged through recipient F1 animals and is still viable. The animal examined for Richter’s-like pathology in this report, mouse 84.1 (Fig. 1, star), was the eighth generation of the passaged clone. At that point, the line had been carried for 4 years. It should be stressed that this conversion to a Richter’s-like phenotype is not an inevitable consequence of multiple passage through recipient F1 mice. We have in the laboratory a second line established in an old NZB in 1986. This line has maintained CLL-like characteristics through 7 passages in 5 years.

Electron Microscopy of the CLL-like Cell. We first looked for any obvious abnormalities in the hyperdiploid cells by electron microscopy. Peritoneal wash cells were obtained from a year-old NZB with a high percentage of peritoneal hyperdiploid cells and from a normal, age-matched DBA/2 (Fig. 2). The peritoneal wash from the DBA/2 contains a wide variety of cell types, most of which are missing from the NZB. The NZB peritoneum shows replacement of the normal cell population with a large number of mature-appearing lymphocytes with a high nuclear:cytoplasmic ratio. These cells have prominent nuclei which are generally of smooth outline and which display varying degrees of peripheral chromatin condensation, being quite heavy in some but moderate in others. The limited cytoplasm of these cells is dense, with moderate numbers of mitochondria and little rough endoplasmic reticulum visible. There is no evidence of cytoplasmic inclusions. Thus, these cells look like normal resting lymphocytes, with the only obvious abnormality being their prevalence and the lack of other cell types. This is generally what is seen in the examination of CLL cells.

Analysis of the CLL-like Mouse. Analysis of a standard peripheral blood smear from a CLL-like animal showed an increase in circulating lymphocytes with little cytoplasm and...
THE NZB MOUSE AS A MODEL FOR CLL

Fig. 2. Electron microscopy of PWC from an old NZB with hyperdiploid peritoneal cells (A, top) and from a normal, age-matched DBA/2 (B, bottom). Notice the preponderance of lymphocytes in the NZB PWC. × 5000.

large, round nuclei displaying a hyperchromatic chromatin network as well as an absence of detectable neutrophils in the peripheral blood. Smudged cells were also present (Fig. 3). Histological comparison of a spleen from a normal mouse and the spleen of an old NZB with splenic hyperdiploid cells showed clear distinctions. Histology of the CLL-like NZB spleen showed loss of normal architecture due to diffuse monotonous proliferation of small lymphocytes. The distinction between red pulp and white pulp had essentially disappeared, with lymphocytes especially numerous under the capsule. The lymphocytes in the CLL-like NZB spleen were dark staining cells with large smooth nuclei and little cytoplasm (Fig. 4A). The liver of this CLL-like animal appeared to be normal and showed no pathological changes. This is consistent with our standard observation that the CLL-like cells can generally be found in the spleen, peritoneum, and peripheral blood but not in the liver. The lymph node of this animal was rather atypical. It appeared slightly enlarged but had no detectable hyperdiploid cells. Light microscopy (Fig. 4B) showed a total loss of normal architecture due to diffuse, monotonous sheets of round lymphocytes and mast cells.

Analysis of the Richter’s-like Mouse. Hyperdiploid cells have several extra chromosomes and thus have a G1 peak. DNA analysis of tissue from mouse 84.1 (Fig. 1), a passaged (NZB × DBA/2)F1 with an apparent lymphoma, showed populations of hyperdiploid cells in the spleen, liver, and lymph node (Fig. 5, left). By our criteria this animal is categorized as Richter’s-like. The thymus did not possess hyperdiploid cells. The spleen, it should be noted, has two distinct hyperdiploid populations. The hyperdiploid populations of the liver and lymph node appear to have the same DNA content as the first of the two hyperdiploid G1 splenic populations. However, DNA analysis of an earlier animal in this same lineage, the first F1 recipient of hyperdiploid NZB CLL-like cells (Fig. 5, right), shows a single hyperdiploid G1 peak that corresponds to the second hyperdiploid G1 peak in the Richter’s-like spleen. The most obvious interpretation of this would be that the transformation to a Richter’s-like phenotype was associated with the specific loss of several extra chromosomes carried by the original hyperdiploid clone. The CLL-like clone is still present in the Richter’s-like animal and has retained its original hyperdiploid DNA content but is still present only in the spleen. The second transformation event to a Richter’s-like phenotype allowed metastasis of the new clone into the liver and lymph node. Surface staining of the Richter’s-like spleen, shown in Fig. 6, defines a large population of cells positive for both Ly1 and IgM. The percentage of these double-positive (i.e., CD5+ B) cells is equal to the percentage of hyperdiploid cells determined by DNA analysis. Therefore, the hyperdiploid cells maintain their CD5+ B-cell phenotype over constant passage and through a second transformation event.

Visually, the spleen of the Richter’s-like mouse was grossly enlarged. Microscopic examination shows that normal splenic architecture is completely obliterated. There is a mixture of large cells with cleaved nuclei and prominent nucleoli plus smaller cleaved lymphocytic cells (Fig. 4C). The lymph node was also grossly enlarged. All normal lymph node architecture is obscured (Fig. 4D) by a similar mixture of large cells with cleaved nuclei and prominent nucleoli and small cleaved lymphocytes. The liver, which was apparently unaffected in the CLL-like mouse, had a lumpy appearance in the Richter’s-like animal. Unlike the spleen and lymph nodes, the liver did not appear enlarged. This animal had infiltration of the hepatic parenchyma and the portal triads with a combination of large cleaved cells and small lymphocytes such as were seen in the spleen and lymph node. Thus, the histology of the Richter’s-
like mouse is quite distinct from that of the CLL-like mouse, although both animals are displaying hyperplasias of hyperdiploid CD5+ B-cells.

Immunoglobulin Heavy Chain Variable Gene Usage. In order to establish the relationship between the CLL and Richter's-like clones, we examined the immunoglobulin gene usage of both Richter's-like and CLL-like cells. The mice examined were described in Fig. 1; mouse 13.2 is CLL-like, mouse 25.1 is the first Richter's-like animal, and mouse 65.4 is also Richter's-like. Splenic tissue from 25.1 and 65.4 contained both CLL-like and Richter's-like cells. Fig. 1 shows that the PCR product of the immunoglobulin heavy chain variable (IgH-V) region from the spleens of all three mice examined hybridizes to a probe specific for the murine heavy chain gene family S107. These samples do not exhibit positive hybridization to probes for other murine Ig VH gene families J558, 36-09, 36-60, X24, VGAM3.8, J606, Q52, 7183, VH11, or VH12 (not shown). NZB 9 is a member of a distinct lineage and is shown as a negative control. The S107 family consists of only four members, one of which is a pseudogene (25). The likelihood of a distinct, nonrelated lymphoma using a member of the S107 gene family is thus very remote. Based on this, it seems that in this case the Richter's-like cell is derived from a continued transformation of the CLL-like clone.

DISCUSSION

There are other reports of animal models for CLL present in the literature. There are several murine Ly-1+ B-cell lymphomas, including the spontaneous BCL1 tumor of old BALB/c mice (26, 27) and the inducible CH lymphomas of B10H2aH14b/Wts(2a4b) mice (28), and nude mice have been used to propagate Epstein-Barr virus-transformed human CLL cells (29). The present examination introduces the aged NZB mouse and the (NZB x DBA/2)F1, recipient of hyperdiploid NZB as a model for CLL. It is known that essentially all old NZB mice experience the clonal expansion of a hyperdiploid CD5+ B-cell population (30). These mice also exhibit severe hemolytic anemia, a common event in CLL. This report shows that aged NZB mice display several classic features of CLL pathology. Electron microscopy showed a mature-appearing lymphocyte with no obvious abnormalities. The peripheral blood smear detected an increase in circulating lymphocytes. Smudged cells were also present in the peripheral blood smear. Smudged cells, although not diagnostic, are a standard finding in human CLL, and their presence in these mice is quite noteworthy. Light microscopy of the spleen showed infiltration by small round lymphocytes with large nuclei. This infiltration, most notable under the capsule, resulted in the loss of the clear distinction between the red pulp and the white pulp. These mice develop splenomegaly, resulting from infiltration of the white pulp by the leukemic lymphocyte and subsequent hyperplasia of endogenous lymphocytes. The liver of the CLL-like animal appeared to be unaffected. The lymph node of the CLL-like animal examined here did not have any detectable hyperdiploid cells, but it was enlarged (an unusual observation in the CLL-like mice), and the normal architecture had been obscured by
We also examined an animal with a murine version of Richter's syndrome. Continued passage of the clonal line through successive F1 recipients resulted in a secondary transformation event with subsequent metastasis to the lymph node and liver. The pathology of this malignancy is quite distinct from that seen in the CLL-like NZB. This difference is consistent with the pathology of Richter's syndrome, i.e., obliteration of normal tissue architecture secondary to massive infiltration of the spleen, lymph nodes, and liver by large cells with cleaved nuclei and prominent nucleoli. There are also many small, round cells seen in the liver, spleen, and lymph nodes of the Richter's-like animal that could be either residual CLL-like cells or endogenous lymphocytes. This can be determined by examination of the DNA content of each tissue. The Richter's-like clone is hyperdiploid, but the DNA content is less than that seen in the original CLL-like clone. The spleen of the Richter's-like animal had two distinct hyperdiploid peaks. One of these, the one which displayed the larger DNA content, appeared to match the hyperdiploid peak of the original CLL-like cells. The second hyperdiploid peak seen in the Richter's-like spleen had less DNA than the original CLL-like clone. This second peak has the same DNA content as the hyperdiploid cells detected in the liver and lymph node. Only the spleen of the affected animal showed a hyperdiploid DNA peak that matched the original animal. The evolution to a Richter's-like phenotype seems to have been associated with a specific loss of DNA from the CLL-like clone. The spleen of the affected animal showed a hyperdiploid DNA peak that matched the original animal. The small cells seen in the affected lymph node and liver must therefore be endogenous lymphocytes.

The appearance in a passaged F1, CLL-like animal of an overt malignancy that is hyperdiploid and displays the characteristic cell surface markers of a CD5+ B-cell strongly implies that this second malignancy is derived from continued transformation of the passaged CLL-like clone. Analysis of immunoglobulin heavy chain gene usage in spleens from both CLL-like and Richter's-like animals showed that a member of the small S107 gene family was the only immunoglobulin heavy chain variable region gene expressed. It is unlikely that two distinct malignancies using the same small immunoglobulin variable gene family would arise. Still, the present analysis cannot unconditionally confirm a clonal derivation for these Richter-like cells from the passaged hyperdiploid clone. It is clear that the human diseases are often clonally related. Recently, Miyamura et al. (12) have shown a case of Richter's syndrome and CLL which had identical heavy chain gene rearrangements but distinct surface expression of Ig light chains, indicating a common ancestry for the histologically distinct malignancies. Schots et al. (13) showed a case of Richter's syndrome which was surface Ig-negative (due to a deletion of the Cμ gene) but had an Igα DNA rearrangement identical to that of the original CLL. The derivation of the murine Richter's-like syndrome presented here is still being investigated.

If the Richter's-like lymphoma does prove to be a nonrelated lymphoma, then the possibility that the passaged CLL-like cells have influenced the transformation of a distinct recipient CD5+
B-cell needs to be examined. In humans, Richter’s can clearly result from a neoplastic event distinct from the original leukemia transformation (15–17). One possibility is that a second lymphoma is more easily able to establish itself in the immunocompromised CLL patient. It is also possible that the CLL clone influences the development of the second malignancy in a previously normal lymphocyte. There is abundant evidence of the immunoregulatory ability of the CD5+ B-cell. CD5+ B-cells both in CLL and in multiple myeloma (where the malignant cell is not a CD5+ B-cell) have been shown to be capable of influencing in vitro immune responses. Both positive and negative regulation of B-cell activity by human CD5+ B-cells has been reported (31–33). Soluble protein factors have been implicated in this CD5+ B-cell-mediated immunoregulation. In murine systems, normal CD5+ B-cells have also been shown to be capable of enhancing idiotype-specific responses to T-independent antigens both by antiidiotype interactions and by a late-acting B-cell maturation factor(s) (34, 35). This late-acting factor is also a soluble protein(s) which appears to be distinct from IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, and γ-interferon.

NZB-derived CLL-like CD5+ B-cells are certainly capable of influencing their own environment in vivo. Passage and subsequent growth of the clone in an F1 recipient result in the concurrent hyperplasia of endogenous T-cells and macrophages (36). This hyperplasia may be due in part to our recent finding that these CD5+ B-cell clones have elevated expression of IL-10 (37), which is produced by several B-cell lymphomas and acts as a T-cell growth cofactor and a factor for CTL differentiation (38, 39). This raises the interesting possibility that the increased absolute number of T-cells and the inverted CD4:CD8 ratio seen in the peripheral blood of CLL patients (40–44) are due to IL-10 production by the CLL cell. It is not yet known if IL-10 expression in CLL is normal.

In contrast to the murine T-cell hyperplasia described above, passage of the NZB CLL-like cells results in a clear reduction of normal splenic B-cells and a functional suppression of normal B-cell activity as monitored by decreased serum IgM (36). This shows that the CLL-like cells can also induce a suppressive effect. Understanding the mechanism responsible for this observed murine phenomenon could yield insight into the host of immunological abnormalities associated with CLL, which include hypogammaglobulinemia and decreased memory (45–47) and autoimmune reactions to hematopoietic antigens (48).

Certainly there are some differences between the proposed murine model of CLL and the human disease. The lack of involvement of the lymph node and liver in the CLL-like mice is unlike the pattern of distribution seen in humans, who classically develop extensive lymph node involvement and often show infiltration of the hepatic portal in the liver. Clearly, the NZB CLL-like cells examined here do not target these regions. Also, earlier work has shown that NZB hyperdiploid cells cannot be detected by flow cytometry in the bone marrow (49). It seems that the murine disease is “frozen” at a specific stage loosely equivalent to a human CLL stage I/II of the Rai classification (50), i.e., circulating leukemic lymphocytes and splenomegaly without lymph node involvement. Because CLL can remain asymptomatic for a long time (51), patients rarely present before the disease is quite widespread. The murine disease may represent a very early stage of CLL which is not yet disseminated and past which it cannot progress. This “frozen” state is emphasized by the inability of the CLL-like cells to spread beyond the spleen even after the Richter’s-like cells are well established in the lymph node and liver.

The fact that the CLL-like cells cannot be detected in the murine bone marrow does not exclude the bone marrow as a possible site for the initial transformation event. Bone marrow from aged NZB mice does contain precursors of the hyperdiploid cells (18) and is known to show increased stem cell activity (52). This would be in agreement with the multistep model of disease pathogenesis proposed by Foon et al. (4), which states that polyclonal expansion of normal CD5+ B-cells precedes the malignant transformation of a single cell to CLL. This polyclonal expansion and transformation could occur either in the bone marrow or in the periphery.

The importance of a strong animal model for CLL should be emphasized. The genesis of CLL, unlike that of other leukemias, has not been linked to any definable environmental trigger (53). CLL is the only leukemia for which a possible genetic component has been described, but analysis of this genetic component has been hindered by the fact that the disease is normally detected in adults over age 50. This onset in an aged population makes the necessary familial studies virtually impossible. The old NZB mouse offers a genetically controlled environment for the elucidation of the origin of the leukemic CD5+ lymphocyte. The associated autoimmune symptoms of CLL and the increased incidence of autoimmune disorders in relatives of CLL patients are all similar to the disease seen in aged NZB mice. Ten thousand new cases of CLL are diagnosed every year in the United States alone, and 3–5% of these cases will progress to Richter’s syndrome. Although there are some promising therapies available for CLL management, there is much yet to be learned about this disorder, whose ontogeny, genetic linkage, and associated immunological abnormalities are a complete mystery. The old NZB mouse has the potential to yield valuable information on the ontogeny and pathogenesis of the most common Western leukemia.

REFERENCES


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