Brief Definitive Report

CLONAL EXPANSION OF ABNORMAL B CELLS IN OLD NZB MICE

By M. F. SELDIN,* J. CONROY,§ A. D. STEINBERG,* L. A. D'HOOSTELEARE,‡ AND E. S. RAVECHE§

*From the *Laboratory of Cellular Immunology, National Institute of Arthritis, Musculoskeletal and Skin Diseases; the ‡Laboratory of Genetics, National Cancer Institute, National Institutes of Health, Bethesda, Maryland; and the Department of Microbiology and Immunology, Albany Medical College, Albany, New York*

NZB mice spontaneously develop a B cell–mediated autoimmune disease (1). As these mice age, they develop increased numbers of cycling cells in their spleens followed by hyperdiploidy (2). The hyperdiploid cells are Thy-1$^-$ and stain weakly with anti-Ig (2). These cells may be the origin of the poorly characterized lymphoid malignancies reported in aged NZB mice (3). In addition, NZB mice are reported to have increased numbers of Ly-1$^+$ B cells, which are thought to be a unique lineage responsible for autoantibody production (4). We undertook the present study to examine the molecular and cellular characteristics of NZB hyperdiploid cells in an attempt to elucidate the relationship between these apparently related phenomena. The data indicate that unique Ig gene rearrangements characterize the hyperdiploid cells of individual mice and that they may represent clonal expansion of B cells capable not only of dominating the original host, but also of growing in semiallogeneic recipients.

Materials and Methods

Identification of Hyperdiploid Cells. Ethanol-fixed spleen cell suspensions were stained with a DNA-specific stain, propidium iodide, as previously described (2). Chromosome analysis of NZB hyperdiploid cells and identification of NZB marker chromosomes present in all NZB mitotic cells was performed as previously described (2). Mice used in this study were NIH-derived NZB, DBA/2, and (NZB × DBA/2)F_1. Spleen cell transfusions were performed by intravenous injection of 2×10^7 cells; recipients were analyzed at least 6 wk after transfusion.

Molecular Techniques. DNA was isolated from spleens using standard techniques, digested with restriction endonuclease enzymes (Boehringer Mannheim Diagnostics, Inc., Indianapolis, IN) and 10 μg/sample electrophoresed in 0.9% agarose gels. DNA was transferred to Nytran membranes (Schleicher & Schuell, Keene, NH), hybridized at 65°C, and washed under stringent conditions, all as previously described (5). Ig heavy chain genes were detected using JH_1 and JH_0 (6) inserts kindly provided by Dr. J. F. Mushinski. κ chain genes were detected using insert from pEC_k (5) and V105 (V_{k1} and J_k) (7) or pC3386 (V_{k10}) (7) to detect V_{K10}-specific gene rearrangements. 86T1 was used to detect TCR-β rearrangements (5). All probes were labeled by the hexanucleotide technique with α-[^{32}P]dCTP (3,000 Ci/mmol, Amersham Corp., Arlington Heights, IL) using a Pharmacia Fine Chemicals (Piscataway, NJ) oligolabeling kit and protocol.

Analysis of Cellular Characteristics. Simultaneous fluoresceinated cell surface marker and propidium iodide staining were performed on ethanol-fixed cells. mAbs used were Ly-1 (Becton Dickinson & Co., Mountain View, CA), anti-IgM (Tago Inc., Burlingame,

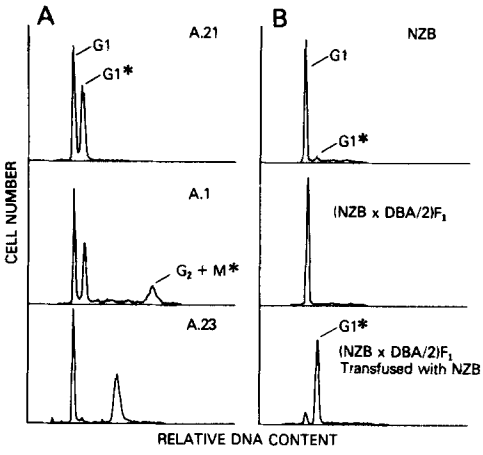

FIGURE 1. (*A*) Representative flow cytometric profiles of spleen cells from three different 1-yr-old NZB mice stained with propidium iodide. The *x*-axis is log scale fluorescence due to DNA content and the *y*-axis is relative cell number. For each individual spleen sample 10,000 cells were analyzed. (*B*) A representative single experiment. The top panel is the DNA profile of the donor NZB spleen cells. The first large peak represents cells with the normal G_1 content of DNA, the next smaller peak consists of hyperdiploid G_1 cells with increased DNA content ($G1^*$). The middle panel is an unmanipulated 4-mo-old (NZB × DBA/2)F_1 mouse with the normal 2C amount of DNA. The bottom profile is from the spleen of an (NZB × DBA/2)F_1 mouse transfused 6 wk earlier with the NZB spleen cells shown in the top panel. The smaller first peak represents F_1 normal G_1 cells; the larger second peak consists of hyperdiploid donor NZB G_1 cells (G_1^*) with increased DNA content.

CA), anti-Ia (New England Nuclear, Boston, MA), and 14.8 anti-Ly-5 (B220) kindly provided by Dr. T. M. Chused. Ly-1 B cells were stained using a biotinylated anti-Ly-1 (Becton Dickinson & Co.) followed by phycoerythrin-avidin (Becton Dickinson & Co.) and FITC-labeled anti-IgM (Tago Inc.). Intracellular p53 protein was stained in ethanol-fixed cells with an anti-p53 (clone 1421) (8) followed by a FITC-labeled goat anti–rat Ig (Cooper Diagnostics, Malvern, PA) and simultaneous staining with propidium iodide (25 μg/ml). Serum anti-ssDNA and IgM levels were determined by an ELISA technique (9).

Results and Discussion

Proliferative Characteristics of NZB Spleen Cells. >80% of NZB mice >9 mo old possess at least 5% hyperdiploid spleen cells (2). The hyperdiploid cells frequently have additional chromosomes 10, 15, 17, and X (2), none of which contain members of the Ig gene family. Fig. 1*A* illustrates flow cytometric analyses of DNA content of three representative spleen cell populations from individual aged NZB mice. All three profiles show a large percentage of hyperdiploid cells (G_1^*). The middle profile shows a spleen with a large number of proliferating hyperdiploid cells (G_2 + M*), which is characteristic of about half of hyperdiploid NZB spleens. The bottom profile of panel *A* is that of an NZB mouse that possessed tetraploid cells as confirmed by cytogenetic analysis. The hyperdiploid cells were not able to be grown *in vitro* in the presence of EL-4 supernatants or ssDNA as a source of antigen stimulation. However, the hyperdiploid cells can be passaged into non-irradiated (NZB × DBA/2)F_1 recipients where they can be identified as of NZB origin because of unusual C-banded marker chromosomes (2). Fig. 1*B* demonstrates the enhanced proliferative capacity of NZB hyperdiploid spleen cells in the F_1 recipients. Mice transfused with NZB spleen cells containing both normal and hyperdiploid cells were populated with only NZB hyperdiploid cells (data not shown). Although these mice developed splenomegaly, and hyperdiploid cells were found in low numbers in the peripheral blood, there was no evidence of infiltration into any other organ and transfused recipients lived for >1.5 yr. Thus, although hyperdiploid cells have an abnormal growth potential they do not behave as frank lymphomas.

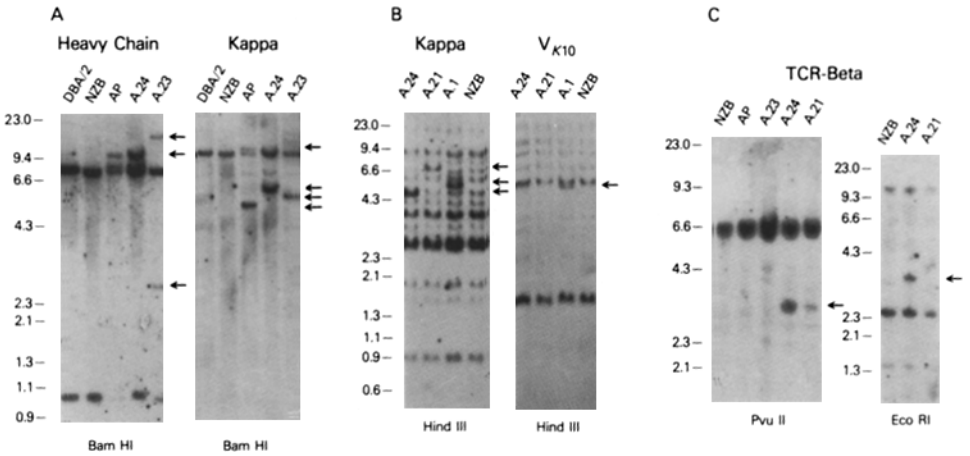

FIGURE 2. Identification of Ig and TCR rearrangements in hyperdiploid spleens. NZB is a DNA sample from a 1-yr-old NZB mouse without hyperdiploid cells. AP represents analysis of DNA obtained from a (NZB × DBA/2)F_1 mouse transfused with NZB hyperdiploid cells and A.23, A.24, A.21, and A.1 are DNA samples from individual 1-yr-old NZB mice with hyperdiploid cells. Arrows indicate non-germline bands and molecular weight markers are indicated to the left of individual blots. (*A*) Clonal rearrangements of heavy chain genes (JH, and JH_0) (6) and κ light chain genes (pEC_k) (5) in hyperdiploid cells can be detected in DNAs digested with the Bam HI restiction enzyme. (*B*) The results of a single gel in which Hind III–digested samples were probed with a V_{K1} probe (V105) that contains J chain sequences (7) and thus should detect all V_κ rearrangements (*left*) and PC3886, a V_{K10}-specific probe (7) (*right*). In panel C the Pvu II digest probed with 86T1(5) detects both TCR-β1 and TCR-β2 rearrangements, whereas the Eco RI digest detects only TCR-β1 rearrangement.

Molecular Analysis of Hyperdiploid Cells. To examine the cellular origin of hyperdiploid cells, DNA from individual hyperdiploid and nonhyperdiploid spleens were analyzed by Southern hybridization with probes to detect Ig gene rearrangements. Fig. 2*A* shows discrete bands not in a germline configuration indicative of rearrangements of both V_H and V_κ genes in both individual aged NZB (A.24, A.23) and transfused F_1 mice (AP). All of five individual hyperdiploid spleens demonstrated these clonal rearrangements. None of these mice had rearrangements of lambda light chain genes (data not shown). None of six nonhyperdiploid NZB spleens demonstrated discrete non-germline bands. In addition MRL-*lpr/lpr* mice neither demonstrated detectable hyperdiploid cells in their spleens nor had additional bands detected on Southern blot analysis (data not shown). These results indicate that hyperdiploid cells represent the clonal expansion of a single or a few B cells in NZB mice. However, the presence of clonal immunoglobulin gene rearrangements is not a general feature of autoimmunity.

Probes specific for V_H and V_κ gene families were used in an attempt to determine if preferential usage of specific variable region gene families was a property of hyperdiploid cells. In Fig. 2*B* one (A.1) of three individual spleen preparations demonstrated a V_{K10} clonal rearrangement. However, by this analysis V_{K10} was not uniformly used by these clonally rearranged B cells. Other V_H (7183, S107, Q52) and V_κ (V_{K8}, V_{K21})-specific probes did not reveal novel bands or germine deletions. Preferential use of V_{H7183} in rheumatoid factor and anti-DNA autoantibodies has been reported (10). Other groups have not observed a

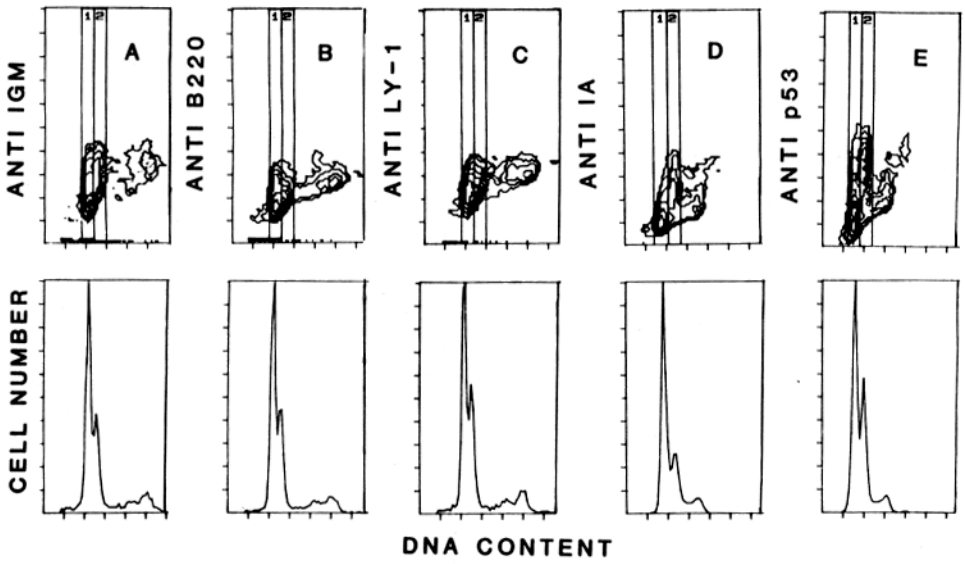

FIGURE 3. Dual parameter flow cytometric analysis of spleen cells from (NZB × DBA/2)F_1 mice transfused with hyperdiploid cells. In each panel (*A–E*) a dual parameter histogram is depicted with the *x*-axis representing DNA content and the *y*-axis (each mark represents 100 U) representing expression of the indicated cell surface marker, except in *E* where intracellular p53 protein is measured. Below each dual parameter histogram is a single parameter histogram with the *x*-axis representing fluorescence due to DNA content and the *y*-axis cell number. In each panel the cells in area 1 represent normal 2C cells (plus some spill over from the hyperdiploid cells); those in area 2 are predominantly hyperdiploid cells with increased DNA content. The cells to the right of area 2 represent proliferating hyperdiploid cells since untransfused F_1 mice have undetectable numbers of proliferating cells. Brightly staining cells are those with >240 U on the *y*-axis.

similar preference (11). The current study does not provide evidence for specific use of variable region gene families by clonally expanded NZB B cells; cloning studies are in progress to clarify this question.

Because some B cell lymphomas demonstrate rearrangements of TCR genes, an analysis of TCR-β genes was performed. In Fig. 2*C*, one hyperdiploid spleen (A.24) of five studied exhibited a TCR-β, rearrangement. Thus, TCR rearrangement is not a general characteristic of these clonally expanded abnormal cells.

Cellular Characteristics. Experiments were next performed to examine the cell surface characteristics of hyperdiploid cells. Fig. 3 shows the results of representative experiments in which spleen cells of (NZB × DBA/2)F_1 recipients of hyperdiploid cells were examined by flow cytometry. Hyperdiploid cells were found to be IgM-bright (*A*), Ly-5^+ (B220) (*B*), Ly-1^+ (intermediate staining) (*C*), IgD-dull (data not shown), and THB-dull (data not shown). Transfused recipients with >50% hyperdiploid cells were found to have elevated percentages of Ly-1 B cells relative to untransfused F_1 mice (means of 20.6 and 4.0, respectively). These cell-surface characteristics are similar to those proposed for a lineage of autoantibody-producing B cells (4). In addition, hyperdiploid-transfused F_1 recipients also had modest elevations in anti-ssDNA antibodies and serum IgM levels when compared with untransfused F_1 mice, but not nearly in the amount present in aged NZB mice (Nicolli et al., manuscript in preparation). Thus it is

unclear whether hyperdiploid cells might arise from autoantibody-producing B cells.

Both surface Ia expression and intracellular p53 protein are increased in activated lymphoid cells. Elevation of p53 also has been implicated in transformation (9). Hyperdiploid cells showed increased staining with both anti-Ia and anti-p53 (Fig. 3). The current study failed to show evidence of rearrangement or gene amplification of p53, c-*myc*, c-*myb*, c-*src*, c-*fos*, c-*raf*, or c-*bas* and thus the mechanism of increased activation and proliferation of hyperdiploid cells remains uncertain.

It appears that hyperdiploid cells arise in conjunction with the same abnormality that results in autoreactive B cells. In support of this idea, no NZB.*xid* mice (0/18) had detectable hyperdiploidy or demonstrated elevated autoantibodies reactive with ssDNA. These results also suggest that the hyperdiploid cells might derive from mature B cells since *xid* interrupts terminal B cell maturation.

NZB hyperdiploid cells appear to represent an intermediary state between autoimmunity and B cell malignancy. An analogous human subpopulation of B cells, Leu-1 B cells, has been implicated both in development of autoimmunity and lymphoproliferative disorders (12, 13). In both the human Leu-1 B cell and the NZB hyperdiploid B cell, surface Ig is of the IgM isotype and the accompanying autoimmune disease is characterized by IgM autoantibodies. Hyperdiploid B cells may represent an expansion of a distinct lineage of B cells, Ly-1 B cells, or, alternatively, B cells incapable of Ig class switching, which thereby escape subsequent immunoregulation.

Summary

The spleens of old NZB mice have an abnormal population of B cells with extra chromosomes. These hyperdiploid B cells manifest increased proliferative capacity; they grow in (NZB × DBA/2)F_1 spleens after intravenous injections. Molecular analysis of individual old NZB and F_1 passaged spleens demonstrate that hyperdiploid cells represent a clonal or oligoclonal expansion of B cells. All spleens with at least 10% hyperdiploid cells demonstrated both heavy and κ light chain immunoglobulin gene rearrangements by Southern blot hybridization. None of the hyperdiploid spleens from old NZB mice had lambda rearrangements and only one of five showed evidence of clonal rearrangement of the TCR-β gene. One also had a V_{K10} clonal rearrangement. Elevated p53 oncogene protein was observed in NZB hyperdiploid spleen cells; however, no p53 or other oncogene rearrangements or amplifications were seen. Hyperdiploid cells were IgM-bright, IgD-dull, Ia^+, dull B220, Thy-1^-, and Ly-1-dull. Spleens with hyperdiploid B cells had increased percentages of Ly-1 B cells. The data suggest that hyperdiploid cells in old NZB mice represent clonal expansion of B cells and that they may represent an intermediate stage between autoimmunity and malignancy.

Received for publication 11 June 1987 and in revised form 13 August 1987.

References

1. Theofilopoulos, A. N., and F. J. Dixon. 1985. Murine models of systemic lupus erythematosus. *Adv. Immunol.* 37:269.

2. Raveche, E. S., O. Alabaster, J. H. Tjio, J. Taurog, and A. D. Steinberg. 1981. Analysis of NZB hyperdiploid spleen cells. *J. Immunol.* 126:154.
3. East, J. 1970. Immunopathology and neoplasms in New Zealand mice and SJL/J mice. *Prog. Exp. Tumor Res.* 13:84.
4. Herzenberg, L. A., A. M. Stall, P. A. Lalor, C. Sidman, W. A. Moore, D. R. Parks, and L. A. Herzenberg. 1986. The Ly-1 B cell lineage. *Immunol. Rev.* 93:81.
5. D'Hoostelaere, L. A., E. Jouvin-Marche, and K. Hupp. 1985. Localization of C_T and C_K on mouse chromosome 6. *Immunogenetics.* 22:227.
6. Lang, R. B., L. W. Stanton, and K. B. Marcu. 1982. On immunoglobulin heavy chain gene switching: two 2b genes are rearranged via switch sequences in MPC-11 cells but only one is expressed. *Nucleic Acids Res.* 10:611.
7. D'Hoostelaere, L. A., and D. M. Gibson. 1986. The organization of immunoglobulin variable kappa chain genes on mouse chromosome 6. *Immunogenetics.* 23:260.
8. Milner, J. 1984. Different forms of p53 detected by monoclonal antibodies in nondividing and dividing lymphocytes. *Nature (Lond.).* 310:143.
9. Laskin, C. A., P. A. Smathers, R. Lieberman, and A. D. Steinberg. 1983. NZB cells actively interfere with the establishment of tolerance to BGG in radiation chimeras. *J. Immunol.* 131:1121.
10. Manheimer-Lory, A. J., M. Monestier, B. Bellon, F. W. Alt, and C. A. Bona. 1986. Fine specificity, idiotypy and nature of cloned heavy-chain variable region genes of murine rheumatoid factor antibodies. *Proc. Natl. Acad. Sci. USA.* 83:8293.
11. Shlomchik, M. J., D. A. Nemazee, V. L. Sato, J. V. Snick, D. A. Carson, and M. G. Weigert. 1986. Variable region sequences of murine IgM anti-IgG monoclonal autoantibodies (rheumatoid factors). *J. Exp. Med.* 164:407.
12. Casali, P., S. E. Burastero, M. Nakamura, G. Inghirami, and A. L. Notkins. 1987. Human Lymphocytes Making Rheumatoid Factor and Antibody to ssDNA belong to Leu-1+ B-Cell Subset. *Science (Wash. DC).* 236:77.
13. Hardy, R. R., K. Hayakawa, M. Shimizu, K. Yamasaki, and T. Kishimoto. 1987. Rheumatoid Factor Secretion from Human Leu-1+ B Cells. *Science (Wash. DC).* 236:81.