International Immunology, Vol. 3, No. 8, pp. 839–842

I-E-restricted monoclonal expansion of B lymphocytes in the thymus of NOD mouse

Kenji Watanabe, Reiko Tanaka, Takashi Nishimura, Yoshihiro Kumagai[1], Jun-ichi Miyazaki[2], Ken-ichi Yamamura[2], and **Sonoko Habu**

Department of Immunology, Tokai University School of Medicine, Bohseidai, Isehara 259-11, Japan
[1]Biomaterial Research Institute Co., Ltd, Yokohama, Japan
[2]Department of Genetics, Kumamoto University, Medical School, Kumamoto, Japan

Key words: thymus, lymphoid follicle, NOD mouse, I-E-expressing NOD transgenic mouse, monoclonal expansion

The non-obese diabetic (NOD) mouse has been extensively studied as a model for insulin-dependent diabetes mellitus (IDDM) because the selective destruction of the pancreatic β cells in association with lymphocytic infiltration is similar to that seen in humans (1). Transfer experiments have demonstrated that T cells obtained from the diabetic NOD spleen are capable of inducing IDDM in non-diabetic NOD (2) and nude mice (3), suggesting that IDDM is a T cell-mediated autoimmune disease. Signore *et al.* (4) recently reported that the number of B cells infiltrating the pancreatic islets of the NOD mouse is nearly as great as that of T cells. However, the precise role of B lymphocytes in the development of diabetes has not yet been clarified.

Here we demonstrate that the NOD thymus contains an increased number of B220+ cells co-expressing sIgM and/or sIgD but not sIgG2a or Ly-1. Southern blot analysis of the NOD thymus showed a distinct rearranged band at 3.8 kb in the NOD thymus when extracted DNA were hybridized with a mouse J_H probe. Such a rearranged band, however, did not appear in I-E-expressing NOD transgenic mice, which are protected from developing insulitis or diabetes. These results suggest that monoclonal expansion of thymic B cells in NOD mice occurs in association with a lack of I-E expression, and may be involved in the pathogenesis of IDDM.

Organ specific autoantibodies have been reported in diseases such as myasthenia gravis (5) and Basedow's disease (6). There is also an increased number of thymic B cells in these diseases (7). In addition, these thymic B cells can produce autoantibodies *in vitro* with or without stimulants (8,9). In order to ascertain whether such B cells are present in NOD mice, we investigated thymocytes of NOD mice.

Thymic lymphocytes were obtained from 5- to 18-week-old NOD mice and then stained with monoclonal antibodies against B220 (10). FACScan analysis of the stained cells demonstrated that 0.9–8.1% (mean 3.9%) of the lymphocytes obtained from the thymus of 18-week-old NOD mice were B220+. In contrast, in the younger NOD mice (5–12 weeks old), B220+ cells constituted only 0.1–1.0%, which was essentially the same as the proportion seen in 18-week-old ICR and C57BL/6 mice (data not shown). In addition, the cell number of 18-week NOD thymus was never smaller than that of younger mice (data not shown), indicating that intrathymic B cells in aged NOD mice actually increased. Two-color analysis of the B220+ cells in the 18-week

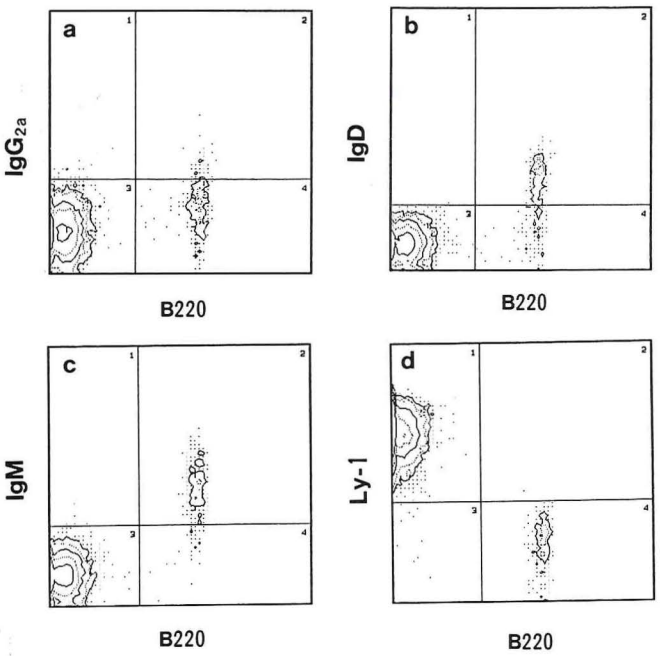

Fig. 1. Two-color analysis of thymocytes showing B220+ cells in 18-week-old NOD mice. B220+ cells constituted 6.8% of the lymphocytes obtained from the thymus. The B220+ IgM+ cells made up 5.5% and B220+ IgD+ cells 3.7%, i.e. the B lymphocytes were composed mainly of B220+ IgM+ IgD+ cells and the remainder mainly of B220+ IgM+ IgD− cells. Methods: NOD mice were provided by Clea Japan Inc. (Tokyo, Japan), and bred under specific pathogen-free conditions. Dissociated lymphocytes from NOD thymus were incubated first with fluorescein-conjugated anti-B220 (RA3 3A1) (10) and next with biotinylated monoclonal antibodies against IgG2a (5.7), IgD (11-6.3), IgM AF6-78) (Pharmingen), or Ly-1 (53-7.313, Becton Dickinson) followed by incubating with phycoerythrin-conjugated streptavidin (Amersham). A total of 10,000 double color labeled cells were analysed with a FACScan flow cytometer (Becton Dickinson).

Correspondence to: S. Habu

Transmitting editor: K. Okomura

Received 26 April 1991, *accepted* 24 May 1991

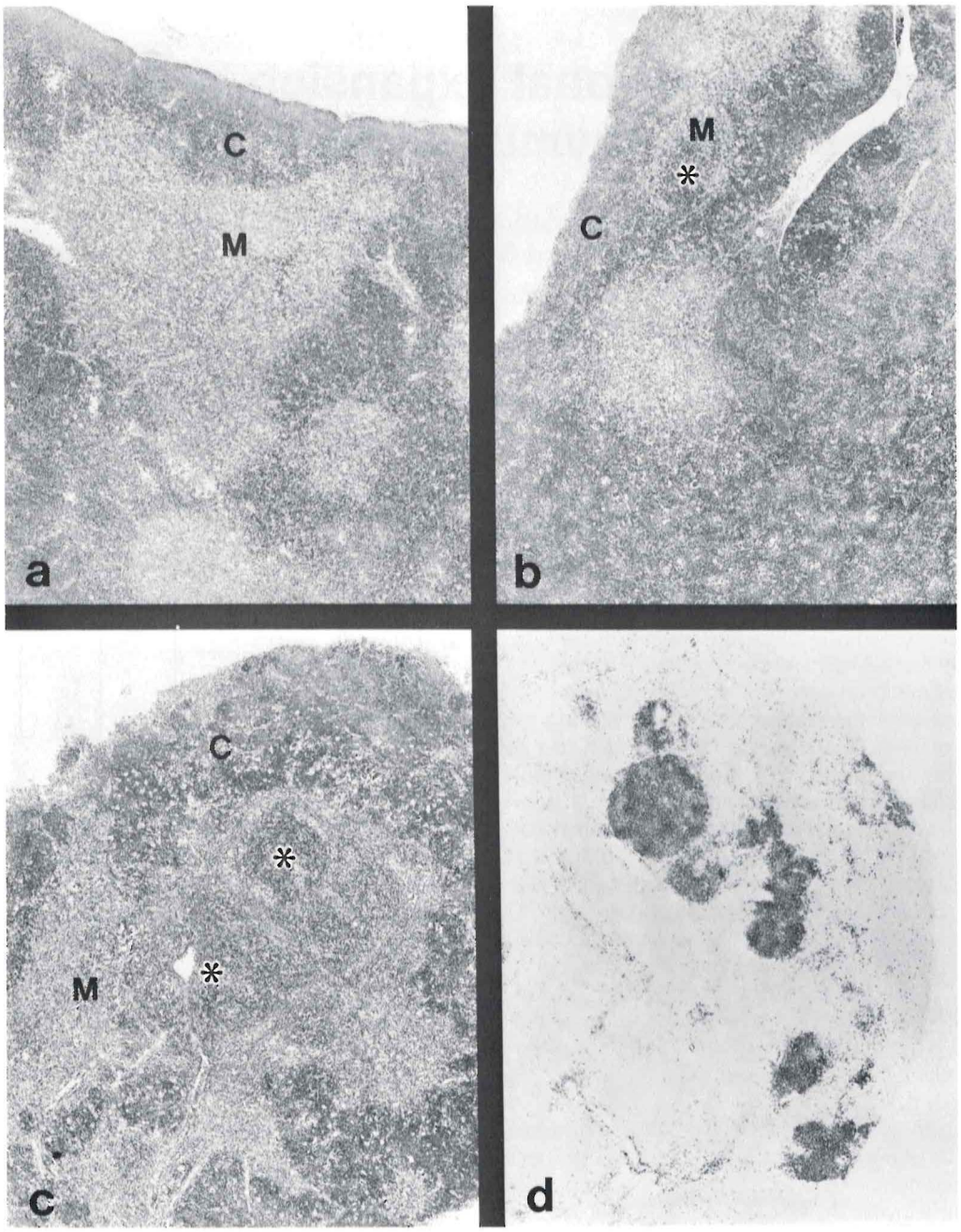

Fig. 2. Lymphoid follicles in the NOD thymus. (a – c) Hematoxylin – eosin staining of the thymus section (×36) showing follicle formation of lymphocytes (*, lymphoid follicle) in the medulla (C, cortex; M, medulla) at 12 (b) and 18 weeks (c), but not at 5 weeks (a). These lymphoid follicles were B220+ (d, 18-week NOD thymus; ×30). Methods: Acetone-fixed 4 μm frozen sections of the 18-week NOD thymus were incubated with biotinylated anti-B220 at room temperature, then incubated with avidin-conjugated horseradish peroxidase (Amersham). For visualization of reaction products, the sections were dipped in Karnovsky's solution.

NOD thymus showed that these cells also express sIgM and/or sIgD but not sIgG2a. As shown by the FACS profiles, the majority of B220+ cells in the 18-week NOD thymus were IgM+ IgD+ (Fig. 1).

There have been reports of the presence of a small population of thymic B cells in several mouse strains (11) as well as in humans (12). Recently Miyaba-Inaba *et al.* (11) reported that thymic B cells contain Ly-1+ B cells, which were originally found in the peritoneal cavity and spleen. They have been reported to be a B cell lineage distinct from ordinary Ly-1− B cells (13). B220+ cells in the 18-week NOD thymus, however, were Ly-1− (Fig. 1). Therefore, the B220+ cells in the NOD thymus may proliferate gradually with aging from ordinary B cells seen in the non-autoimmune mouse thymus.

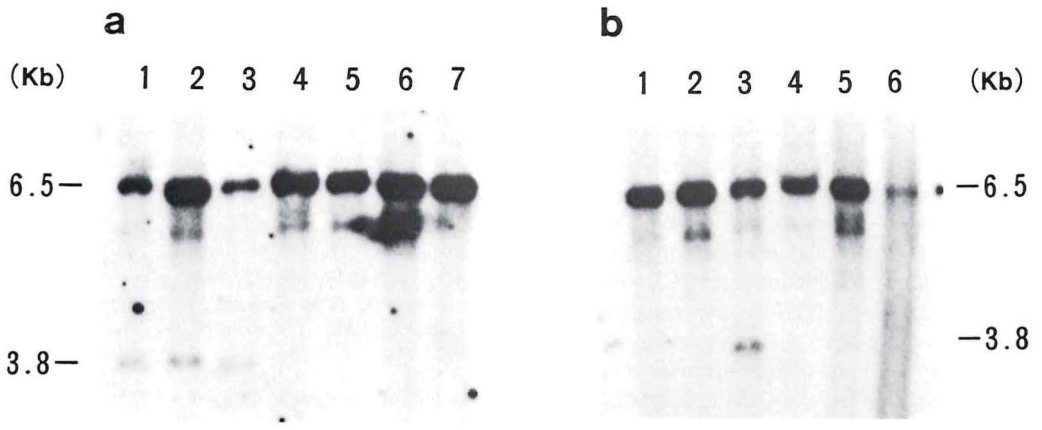

Fig. 3. Southern blot analysis of DNA extracted from thymus of NOD and non-NOD mice. (a) Lanes 1 – 3, 18-week NOD thymus; lane 4, 18-week thymocytes depleted of B220+ cells by FACStar (Becton Dickinson); lane 5, 5-week NOD thymus; lane 6, 8-week NOD thymus; lane 7, 18-week ICR liver. (b) Lanes 1 and 2, 18-week I-E transgenic NOD thymus; lane 3, 18-week NOD thymus; lane 4, 18-week ICR thymus; lane 5, 18-week C57BL/6 thymus; lane 6, 18-week ICR liver. Methods: DNA samples (10 μg) from thymus and liver were digested by restriction enzyme *Eco*RI and submitted for agarose gel electrophoresis. After transfer on a positively charged nylon membrane (Pall Biosupport), it was hybridized with a cloned mouse genomic J_H probe. Sizes of rearranged restriction fragments (in kb) were estimated by comparison with a *Hin*dIII-digested λ-phage DNA.

Table 1. Appearance of B220+ cells and a particular rearanged Igh gene in NOD thymus

	NOD								I-E NOD[b]		
	5 weeks[a]	5 weeks	8 weeks	8 weeks	18 weeks	18 weeks	18 weeks	18 weeks	12 weeks	18 weeks	18 weeks
B220+ cells (%)[c]	0.3	0.5	0.6	0.7	2.8	0.9	1.1	2.0	0.4	1.5	2.0
3.8 kb band[d]	–	–	–	–	+	+	+	+	–	–	–

[a]The proportion of B220+ cells and the presence of rearranged Igh gene in the thymi of eight NOD and three I-E NOD mice from 5 to 18 weeks were summarized.
[b]I-Eα^d gene was introduced in the NOD mouse[18].
[c]The percentage of B220+ cells in whole thymocytes was estimated from FACS analysis.
[d]A discrete 2.7 kb rearranged band on DNA samples digested with *Eco*RI was detected by Southern blotting using a J_H probe (pJ_H34).

Next we examined the distribution of B cells in the thymus. Morphological study of thymus tissue sections showed that the thymi of 12- to 18-week-old NOD mice were hyperplastic and had follicular lymphocyte aggregates initially appearing around 12 weeks and increasing through the 18th week. Such aggregates of lymphocytes were not seen in 5- to 8-week-old mice (Fig. 2). The follicular structures were located mainly in the medulla near the cortico-medullary junction, suggesting that these lymphocytes had proliferated possibly in response to local stimuli. To determine whether the follicular lymphocytes correspond to the B220+ cells demonstrated by FACS analysis, frozen sections of the NOD thymus were immunohistochemically stained with biotin-conjugated antibodies and visualized with avidin-conjugated horse radish peroxidase. The follicle-forming cells were demonstrated to be positive for both B220 (Fig. 2d) and sIgs, but were not stained by either anti-Ly-1 or anti-Thy-1 (data not shown). The morphological features of the NOD thymus are similar to those found in the human thymus in autoimmune diseases such as myasthenia gravis and Hashimoto's thyroiditis (7).

Recent studies have shown a variety of Igh gene rearrangements in mouse thymic B cells; the V_H repertoires of thymic B cells are as heterogeneous as those of peripheral B cells (14). To determine whether the increased number of B cells in the NOD thymus is due to polyclonal or monoclonal expansion, DNA extracted from thymus cell suspensions of NOD and non-NOD mice were subjected to Southern blot analysis after digestion with the restriction enzyme *Eco*RI. When DNA transferred on a positively charged nylon membrane was hybridized with a cloned mouse genomic J_H probe (pJ_h34), which included J_H3 and J_H4 (extending the *Bam*HI – *Eco*RI site), thymic DNA from all strains showed a 6.5 kb band, corresponding to germline fragments containing the J_H gene cluster, and a slightly smaller band (~5 kb), corresponding to joined DJ segments of D and J_H genes detected in T cells (15). In addition to these bands, another discrete 3.8 kb band was detectable only in the NOD thymus. Among the NOD specimens examined, the 3.8 kb band was observed only in the 18-week NOD thymus. It was not seen in 5- or 8-week NOD thymic tissues, in which B cell numbers were not as high as in the 18-week-old NOD mouse. When DNA was

obtained from the 18-week NOD thymocytes depleted of B220+ cells by FACStar, the 3.8 kb band disappeared (Fig. 3). These observations indicated that the NOD thymus contains the monoclonal expansion of B220+ cells which express the product of a particular Ig gene. In NOD mice, the I-Eα gene cannot be expressed because the mice have the deletion in the promoter region (16). It has been suggested that the T cell repertoire generated by this deficient expression of MHC class II may prime the autoimmune response. However, there has still been disagreement about the particular use of the TCR repertoire for infiltrating T cells in the pancreas (17,18). In order to determine whether clonal expansion of a particular B cell population in the thymus occurs in association with the unique expression of MHC class II in NOD mice, we examined the DNA configurations of B cells in the thymus of I-E-expressing NOD transgenic mice which were established by direct injection of the Eα^d gene into NOD eggs (19). As shown by Southern blotting in Fig. 3(b), a rearranged band at 3.8 kb was not detected in either 12- or 18-week-old I-E-expressing NOD transgenic mice, although 2.1% of thymocytes were B220+. Since such a rearranged band was observed even in the 18-week NOD thymus which contained only 0.9% B220+ cells, it is unlikely that the appearance of the 3.8 kb band is due solely to the increased proportion of B220+ cells (Table 1). In fact, the total cell number of the 18-week I-E transgenic thymus was ~25% of the thymus cells of the same aged NOD thymus (data not shown), implying that B cells are not increased in the 18-week-old I-E transgenic mouse. Thus, it is hypothesized that clonal expansion of a particular B cell population in the thymus is a specific phenomenon for NOD mice. Furthermore, the B cell expansion may be involved in pathogenesis of IDDM, as the I-E-expressing NOD transgenic mouse develops neither insulitis nor diabetes.

The reverse correlation between I-E expression and clonal expansion of B cells in NOD mice suggests that I-E expression might be responsible for regulating proliferation of a particular B cell clone in NOD mice through interaction with helper T cell clones which have been eliminated in I-E+ but not I-E− mice. Although specific T cell clones interacting with the particular B cell clone have not been yet identified in NOD mice, T cells bearing a TCR repertoire free from I-E restriction may recognize or cross-react with autoantigens, presumably pancreatic β cell components, presented by antigen-processing cells or B cells expressing unusual I-A due to replacement amino acid residues at positions 56 and 57 (20). To determine the antigens reacting with clonally expanded B cells in NOD thymus, we are now on the way to establishing a B cell hybridoma from the old NOD thymus.

Abbreviations

IDDM	insulin-dependent diabetes mellitus
NOD	non-obese diabetic

References

1 Makino, S., Kunimoto, K., Muraoka, Y., Mizushima, Y., Katagiri, K., and Tochino, Y. 1980. Breeding of a non-obese diabetic strain of mice. *Exp. Anim.* 29:1.

2 Bendelac, A., Carnaud, C., Boitard, C., and Bach, J. F. 1987. Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. *J. Exp. Med.* 166:823.

3 Harada, M. and Makino, S. 1986. Immunological manipulation of diabetes production in NOD mice. In Tarui, S., Tochino, Y., and Nonaka, K., eds, *Insulitis and Type I Diabetes—Lesson from the NOD Mice,* p. 143. Academic Press, New York.

4 Signore, A., Gale, E. A. M., Andreani, D., Beverley, P. C. L., and Pozzilli, P. 1989. The natural history of lymphocyte subsets of infiltrating the pancreas of NOD mice. *Diabetologia* 32:282.

5 Patrick, J. and Lindstrom, J. 1973. Autoimmune response to acetylcholine receptor. *Science* 180:871.

6 Manley, S. W., Bourke, J. R., and Hawker, R. W. 1974. The thyrotrophine receptor in guinea-pig thyroid homogenate: interaction with the long-acting thyroid stimulator. *J. Endocrinol.* 61:437.

7 Habu, S., Kameya, T., and Tamaoki, N. 1971. Thymic lymphoid follicles in autoimmune diseases I. Quantitative studies with special reference to myasthenia gravis. *Keio J. Med.* 20:45.

8 Scadding, G. K., Vincent, A., Newsom-Davis, J., and Henry, K. 1981. Acetylcholine receptor antibody synthesis by thymic lymphocytes: correlation with thymic histology. *Neurology* 31:935.

9 Fujii, Y., Monden, Y., Nakahara, K., Hashimoto, J., and Kawashima, Y. 1984. Antibody to acetylcholine receptor myasthenia gravis. *Neurology* 34:1182.

10 Coffman, R. L. and Weissman, I. L. 1981. A monoclonal antibody that recognizes B cells and B cell precursors in mice. *J. Exp. Med.* 153:269.

11 Miyama-Inaba, M., Kuma, S., Inaba, K., Ogata, H., Iwai, H., Yasumizu, R., and Ikehara, S. 1988. Unusual phenotype of B cells in the thymus of normal mice. *J. Exp. Med.* 168:811.

12 Isaacsson, P. G., Norton, A. J., and Addis, B. J. 1987. The human thymus contains a novel population of B lymphocytes. *Lancet* ii:1488.

13 Hayakawa, K., Hardy, R. R., Parks, D. R., and Herzenberg, L. A. 1983. The "Ly-1 B" cell subpopulation in normal, immuno-defective and autoimmune mice. *J. Exp. Med.* 157:202.

14 Andreu-Sanchez, J. L., Faro, J., Alonso, J. M., Paige, C. J., Martinez-A., C., and Marcos, M. A. R. 1990. Ontogenic characterization of thymic B lymphocytes. Analysis in different mouse strains. *Eur. J. Immunol.* 20:1767.

15 Kurosawa, Y., von Boemer, H., Haas, W., Sakano, H., Trauneker, A., and Tonegawa, S. 1981. Identification of D segments of immunoglobulin heavy-chain genes and their rearrangement in T lymphocytes. *Nature* 290:565.

16 Hattori, M., Buse, J. B., Jackson, R. A., Glimcher, L., Dorf, M. E., Minami, M., Makino, S., Moriwaki, K., Kuzuya, H., Imura, H., Strauss, W. M., Seidman, J. G., and Eisenbarth, G. S. 1986. The NOD mouse: recessive diabetogenic gene in the major histocompatibility complex. *Science* 231:733.

17 Reich, E.-P., Sherwin, R. H., Kanagawa, O., and Janeway, C. A., Jr 1989. An explanation for the protective effect of the MHC class II I-E molecule in murine diabetes. *Nature* 341:326.

18 Nakano, N., Kikutani, H., Nishimoto, H., and Kishimoto, T. 1991. T-cell receptor V usage of islet β cell-reactive T cells is not restricted in non-obese diabetic mice. *J. Exp. Med.* 173:1091.

19 Uehira, M., Uno, M., Kurner, T., Kikutani, H., Mori, K., Inomoto, T., Ueda, T., Miyazaki, J., Nishimoto, H., Kishimoto, T., and Yamamura, K. 1989. Development of autoimmune insulitis is prevented in Eα^d but not in Aβ^k NOD transgenic mice. *Int. Immunol.* 1:209.

20 Archa-Orbea, H. and McDevitt, H. O. 1987. The first external domain of the non-obsese diabetic mouse class II I-A β chain is unique. *Proc. Natl Acad. Sci. USA* 84:2435.