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The influence of thymic abnormalities on the development of autoimmune diseases

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Summary. The relationship between thymic abnormalities and development of autoimmune diseases was studied in MRL/1 and NZB/NZW F1 (NZB/W F1) mice. Thymic abnormalities, including plasma cell infiltration into the thymus, were observed in 25% of MRL/l mice as early as 1 mo and in all mice after 2.5 mo. The thymic abnormalities preceded both infiltration of lymphoid cells into the kidney and salivary glands, and also preceded an increase in the titer of circulating immune complexes (CIC). When MRL/1 and NZB/W F1 mice were divided into two groups for each strain at the critical age when the mice had begun to show thymic abnormalities, the group with abnormal thymuses showed more marked pathological findings in other organs and a higher level of CIC than the group with more normal appearing thymus. In addition, the group with abnormal thymus demonstrated lower responsiveness of their lymphocytes in mixed-lymphocyte culture than the group with normal thymus. Thymus grafts from donors of autoimmune or non-autoimmune strains into nude mice revealed that thymic functions, reconstitutive of immunologic parameters of nude mice, are rapidly lost with age. These results suggest that morphological and functional abnormalities of the thymus are involved in the pathogenesis of autoimmune diseases.

Introduction

It has been demonstrated that patients with autoimmune deseases such as systemic lupus erythematosus (SLE) show decreased T cell functions [1, 2]. The availablitity of several murine strains with SLE-like disease has prompted extensive studies concerning the immunological abnormalities of these mice in efforts to understand better the fundamental nature of autoimmune diseases. Mice of autoimmune prone strains show premature thymic atrophy, T cell dysfunction, B-cell hyperactivity, production of autoantibodies, elevation of circulating immune complexes (CIC), and immune complex (IC)-type glomerulonephritis [3,4]. Immunological abnormalities of autoimmuno-prone mice have been attributed to T cells, B cells, macrophages or stem cells [5–8]. Recently, MRL/l as well as NZB/NZW F1 (NZB/W F1) mice have also been shown to have T cell dysfunction and thymic abnormalities [9–11]. In NZB/W F1 mice, neonatal thymectomy accelerates autoimmune diseases, and it has been thought that the acceleration of autoimmune diseases results from the removal of suppressor T cells by neonatal

thymectomy [12–17]. On the other hand, in MRL/1 mice, it has been reported that neonatal thymectomy prevents autoimmune deseases and lymphoma [18, 19]. It is thought that the lymphadenopathy of MRL/l mice results from massive proliferation of helper T cells, and that prevention of lymphoma and autoimmune diseases by neonatal thymectomy is due to removal of abnormally functioning helper T cells [20]. Consequently, the crucial role of the thymus in the development of autoimmune diseases requires reevaluation. Therefore, we examined the relationship between thymic abnormalities and development of autoimmune diseases at a critical age (1 to 2 mo in MRL/l mice and 6 to 7 mo in NZB/W F1 mice). The present study shows that thymic abnormalities precede both the infiltration of lymphoid cells into the kidney or salivary glands and an increase in the concentration of CIC. In addition, we have found that grafts from thymus of 3 mo old MRL/l mice, unlike the thymus grafts from three mo old mice of certain autoimmune-resistant strains or thymus grafts from newborn MRL/l mice, lack capacity to induce functional T cells in nude mice.

Materials and methods

Animals. Inbred MRL/M-lpr/lpr (MRL/l), MRL/MP-+(MRL/n), BALB/c nu/nu, C3H/HeJ, BALB/c and C57BL/6J mice were obtained from the Jackson Lab., Bar Harbor, Maine, and were maintained under specific pathogen-free conditions in our facilities. NZB/W F1 mice were obtained by mating NZB females with NZW males in our colony. Only female mice were used in this study.

Cell separation. Mice were sacrificed by cervical dislocation. The spleens were removed aseptically, minced, and gently passed through a fine mesh stainless-steel sieve into phosphate-buffered saline (PBS).

Cytotoxic test: Spleen cells which were suspended in TC-199 (GIBCO Lab., Grand Island, New York) with 5% fetal calf serum (FCS: Microbiological Associates, Walkersville, Maryland) were adjusted to 5×10^6 cells/ml and divided into two aliquots of 50 μl. The cells were incubated with 50 μl of a $1/10^3$ dilution of anti-Thy-1.2 antibody (Clone F7D5, Olack Ltd., Bicester, U.K.) for 30 min at 4°C. The cells were then washed once and resuspended in 100 μl of a 1/10 dilution of rabbit complement (Cederland Lab., Ontario, Canada) previously absorbed with mouse spleen cells. After 30 min incubation at 37°C, the viability of the cells was determined by exclusion of trypan blue dye. The counts were converted to a cytotoxic index by the formula:

$$\text{cytotoxic index} = \frac{\%\ \text{viable cells (C alone)} - \%\ \text{viable cells (AB + C)}}{\%\ \text{viable cells (C alone)}} \times 100$$

where C represented complement and Ab, antibody.

Mitogen response: The mitogenic reactivity was determined by measuring incorporation of ^{3}H-thymidine into DNA. Triplicate cultures were set up in wells of flat-bottom microtiter plates (Corning Glass Works 25860, Corning, New York) each containing 2.5×10^5 cells in 0.2 ml RPMI-1640 Nissui Seiyaku Co., Ltd., Tokyo, Japan) that was supplemented with 2 mM Δ-glutamine (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 5% FCS. The cells were cultured in the presence of 25 μg/ml of phytohemagglutinin P (PHA; Difco Lab., Detroit, Michigan) or 5.0 μg/ml of concanavalin A (Con A; Calbiochem, San Diego, California). (The mitogens were tested at various doses and the dose selected was that giving the optimal responses). The cultures were incubated for 72 h at 37°C in a humidified atmosphere of 5% CO_2 in air. ^{3}H-thymidine (0.5 μ Ci in 20 μl) (New England Nuclear, Boston, Massachusetts) was present during the last 4 h of the culturing period. The ^{3}H counts incorporated into trichloroacetic acid-insoluble material were measured by liquid scintillation counting.

Mixed-lymphocyte culture (MLC). MLC was examined by measuring the incorporation of ^{3}H-thymidine into DNA. Triplicate cultures were set up in 96 well round-bottom microtiter trays (Corning Glass Works 25850), each containing 2×10^5 responder cells and 1×10^5 stimulator cells in a total volume of 0.2 ml RPMI-1640 that was supplemented with 2 mM Δ-glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml, 5% human serum and 2-mercaptoethanol (2-ME; 5×10^{-5} M, Wako Pure Chemical Industries, Waco, Texas). Stimulator cells were treated with 25 μg/ml mitomycin-C for 30 min at 37°C. The cultures were incubated for 96 h in a humidified atmosphere of 5% CO_2 in air. The incorporation of ^{3}H-thymidine into DNA was measured by the same method as described in 'Mitogen response.'

Plaque-forming cell (PFC) assay. The PFC response to sheep red blood cells (SRBC) was performed by a method previously described [2].

Thymus graft: Thymus grafting was performed by implanting one to two thymic lobes of MRL/l mice under the kidney capsule of BALB/c nu/nu mice. At 2 mo after grafting, mice were sacrificed, and enlargement of the engrafted thymus was macroscopically and microscopically ascertained. The thymic function was examined by using the spleen cells of thymus-engrafted nude mice.

Measurement of circulating immune complexes (CIC). CIC were detected by using a Clq solid-phase radioimmunoassay (Clq-SPRIA) and a con-glutinin solid-phase RIA (Kg-SPRIA). The Clq-SPRIA was performed by the method of Hay *et al.* [22] with a minor modification using polyvinyl chloride microtiter plates (Dynatech, Alexandria, Virginia). Conglutinin (Kg) was purified as previously described by Nakai *et al.* [23]. Kg-SPRIA was performed by the method of Casali *et al* [24] with a minor modification using microtiter

plates. In both assays, ^{125}I-Protein A (Pharmacia, Uppsala, Sweden) was used for the detection of IgG class CIC.

Histopathological study. Major organs were obtained at autopsy, and sections were stained with periodic acid-Schiff (PAS) and hematoxylin-eosin (HE). A thymus section in which at least one of the following items was oberved was regarded as abnormal: i) severe cortical atrophy, ii) medullary hyperplasia, iii) infiltration of plasma cells, B Cells and granulocytes, iv) formation of lymphoid follicles. In the kidney, lung, liver, salivary gland, lymph node and thyroid gland, infiltration of lymphoid cells was scored on 0 to 3+: normal, 0; ±, 0.5; +, 1.0; ++, 2.0; +++, 3.0.

Immunofluorescent (IF) study. The thymus and kidney specimens were used for study. These specimens were immediately embedded in optimum cutting temperature (OCT) compound and were frozen in dry ice-aceton. Two μm cryostat sections were examined by direct or indirect IF method using the following antisera: rabbit antimouse IgG_1 (Nordic, Netherlands), FITC-labelled anti-mouse IgG_2 (Meloy Laboratories, Springfield, Illinois) FITC-labelled anti-mouse IgA (Meloy Laboratories), goat anti-mouse IgM (Cappel, Cochranville, Pennsylvania), FITC-labelled anti-mouse C_3 (MBL, Nagoya, Japan), FITC-labelled goat anti-rabbit immunoglobulin (Ig) and FITC-labelled rabbit anti-goat Ig (prepared in our laboratory), and goat-anti-Rauscher gp70 sera (National Institutes of Health, Bethesda, Maryland). The immunological specificity of these monospecific antisera directed against mouse IgG_1, IgG_2, IgA and IgM was confirmed by IF test using the mouse myeloma cells, MOPC 31C (γ_1,κ), MOPC 1 (γ_2a,λ), MOPC 141 (γ_1b,κ), J606 (γ_3,κ), MOPC 511 (α_1,κ), TEPC 183 (μ,κ), which were a generous gift of Prof. Honjo, Kyoto University, Faculty of Medicine.

Statistical analysis: Statistical analysis was performed using χ^2 test.

Results

Light microscopic study. The incidence of histopathological abnormalities in the thymus, salivary gland and kidney of MRL/l mice is shown in Figure 1. Thymic abnormalities, such as cortical atrophy and plasma cell infiltration, were observed in 25% of the mice at the age of 1 mo and in all mice after 2.5 mo. As shown in Figure 2, the infiltration of plasma cells into the salivary gland was found in 25% of the mice at the age of 1 mo and in 75% at the age of 3 mo. By contrast, kidney abnormalities such as perivascular infiltration of lymphoid cells were not observed before 1.5 mo. Thus, abnormalities of the thymus preceded appearance of microscopic lesions in the kidney. Therefore, we attempted to examine the relationship between thymic abnormalities and the development of autoimmune disease. MRL/l (1–2 mo) and NZB/W Fl (6–7 mo) mice were sacrificed at a critical age when they

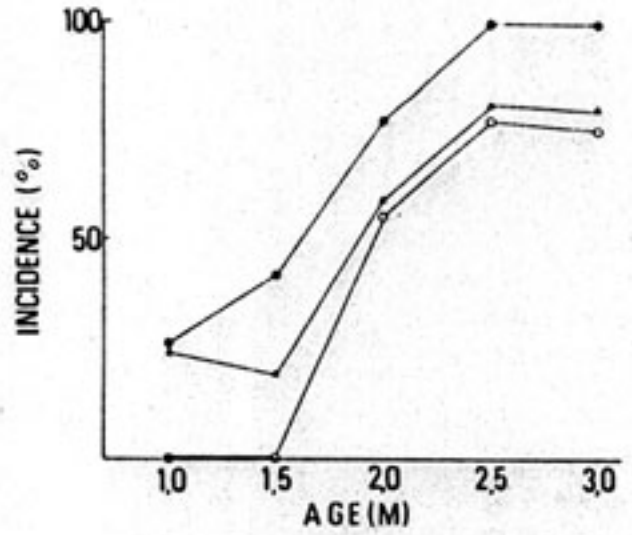

Figure 1. Incidence of histopathological abnormalities of the thymus (●), salivary gland (▲) and kidney (○) of MRL/l mice.

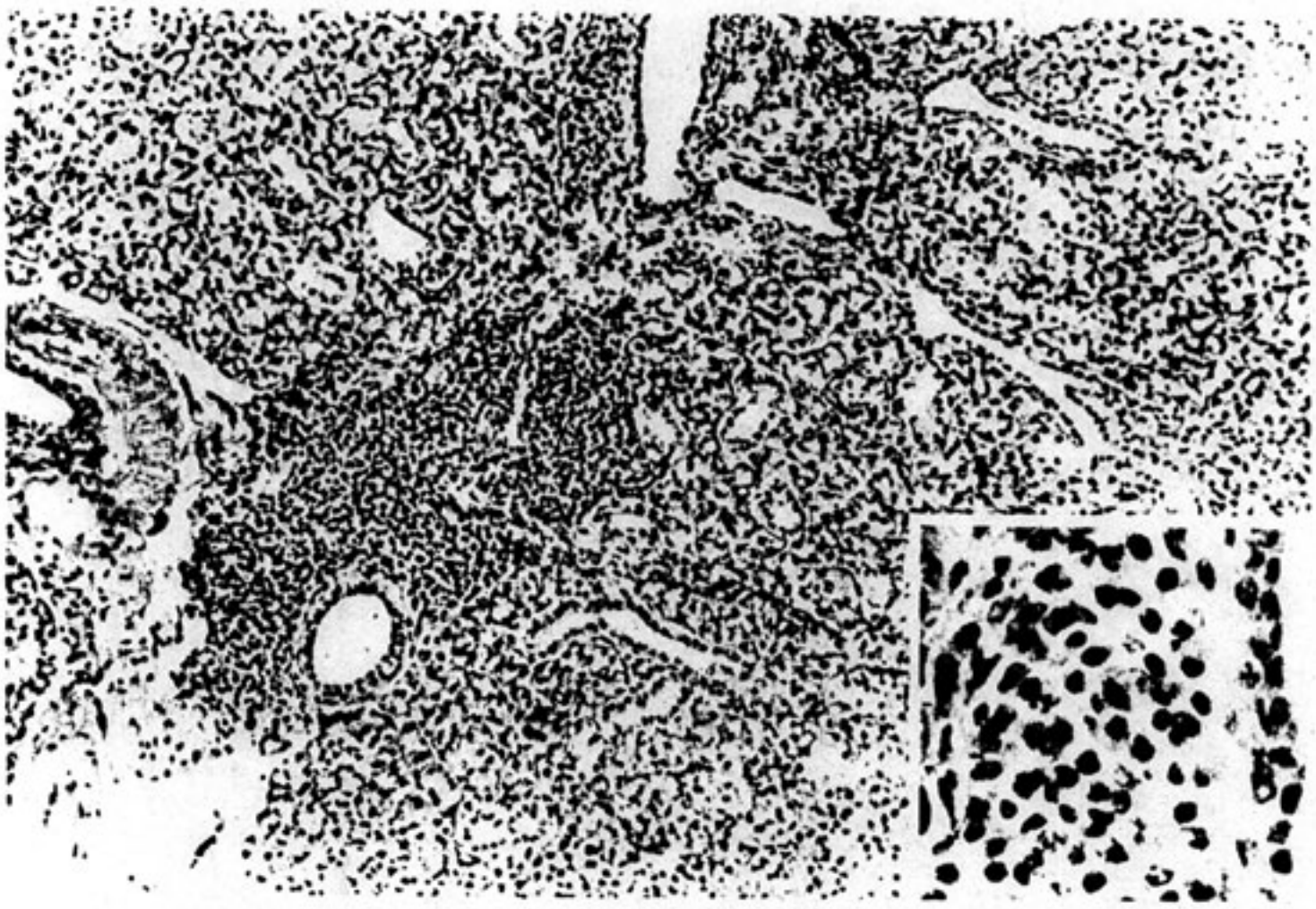

Figure 2. Infiltration of lymphoid cells into the salivary gland of 3 mo MRL/l mice (× 36). The inset shows the remarkable infiltration of plasma cells (× 360).

began to show thymic abnormalities. Mice were divided into two groups according to the presence or absence of thymic abnormalities. As shown in Table 1, the group of MRL/l mice with abnormal thymus demonstrated a significantly higher score of abnormalities in the kidney, salivary gland and lung than the group with normal thymus. In NZB/W F1 mice, the group with abnormal thymus showed a significantly higher score in liver and lung as well as kidney and salivary gland than the group with normal thymus.

Table 1. Relationship between thymic abnormalities and lymphoid cell infiltration into other organs

Mice	Age (M)	Thymus	Score of Pathological Findings[c]			
			Kidney	Salivary Gland	Liver	Lung
MRL/l ♀	1-2	Normal (29)	0.13 ± 0.28	0.33 ± 0.46	0.16 ± 0.27	0.16 ± 0.17
		Abnormal (32)	1.13 ± 0.50[a]	0.73 ± 0.50[b]	0.28 ± 0.28	0.62 ± 0.44[a]
NZB/WF$_1$ ♀	6-7	Normal (5)	0.3 ± 0.1	0.5 ± 0.2	0.2 ± 0.3	0.3 ± 0.1
		Abnormal (7)	2.5 ± 0.2[a]	2.1 ± 0.2[a]	1.3 ± 0.5[b]	1.6 ± 0.3[b]

[a] $P < 0.001$
[b] $P < 0.01$, ( ) Number of mice
[c] Score; −; 0, +; 0.5, +; 1, ++; 2, +++; 3

Study of immunofluorescence (IF). An IF study was performed on the thymus and kidney specimens. IgG_1 and IgA deposits were observed in high incidence in the cytoplasm of mononuclear cells in the abnormal thymus, compared to the normal thymus (Table 2). The glomerular deposits of IgG_1 or IgA and/or C_3 were found in approximately 83% of the mice with abnormal thymus. In these glomeruli, Ig bearing cells were also regularly present in the perivascular regions. By contrast, Ig deposition in glomeruli was found in only 15% of the mice with normal thymus. Ig deposition in glomeruli was mainly distributed in mesangial areas.

Number of T Cells. The relationship between thymic abnormalities and number of Thy-1^+ cells in the spleen was studied in MRL/l mice. As shown in Figure 3, the group with abnormal thymus showed a higher percentage of Thy-1^+ cells in the spleen than did the group with normal thymus; the mean

Table 2. Relationship between thymic abnormalities and IF findings of thymus and kidney in MRL/l mice

Thymus	No. of Positive Mice in IF Findings	
	Thymus	Kidney
Normal	2/13 (15.3%)	2/13 (15.3%)
Abnormal	12/18 (66.6%)	15/18 (83.3%)

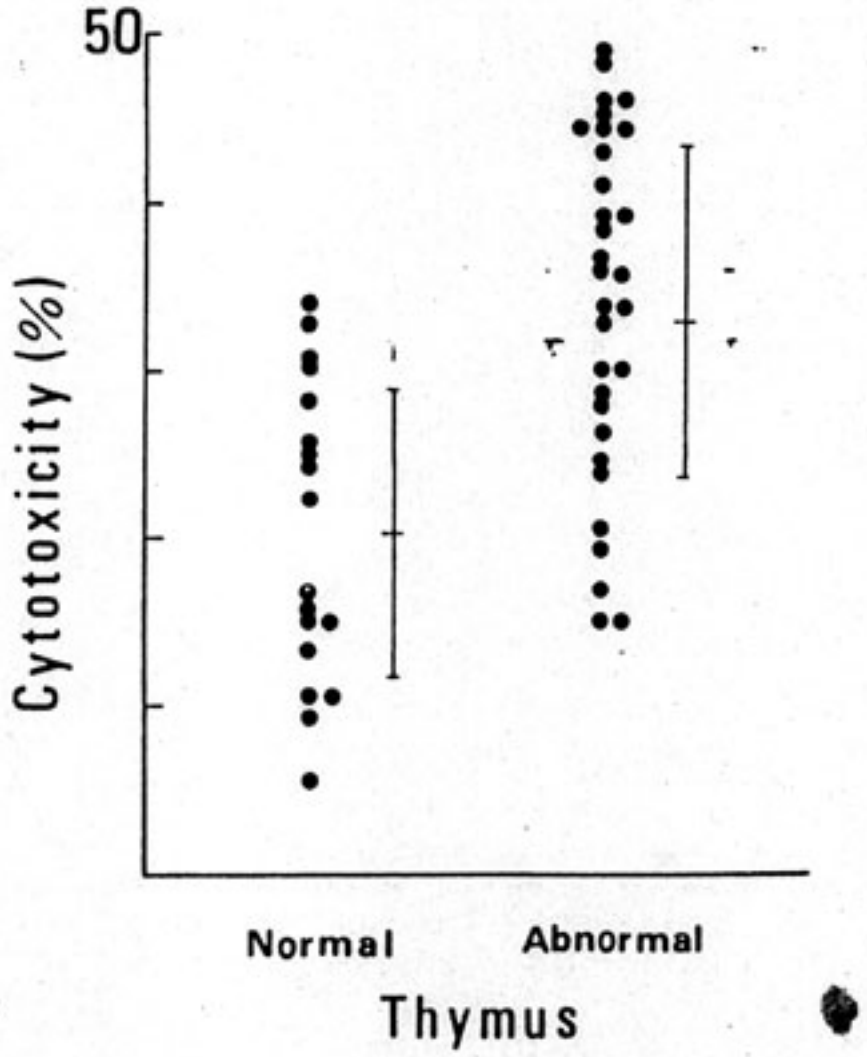

Figure 3. Number of Thy-1^+ cells in MRL/l mice with normal thymus and abnormal thymus ($p < 0.001$).

level of Thy-1$^+$ cells in mice with abnormal thymus was 33.9%, whereas the mean level in mice with normal thymus was 20.5%. This finding indicates that cells with T cell characteristics have proliferated or accumulated in the spleen of the mice with abnormal thymus.

T cell functions. The relationship between T cell functions and thymic abnormalities was studied in MRL/l spleen. Table 3 indicates that the group with abnormal thymus showed a lower responsiveness in mixed-lymphocyte culture (S.I. -1.98) than was observed in the group with normal thymus (S.I. -6.29). With respect to mitogen responses, however, no significant difference was noted between the group with abnormal thymus and that with normal thymus. Therefore, it is likely that the group with abnormal thymus has more Thy-1$^+$ cells in the spleen than the group with normal thymus, whereas some of the cells may have lost their T cell functions, especially alloreactivity as revealed in the MLC response.

Table 3. Relationship between T cell functions and thymic abnormalities in MRL/l mice

Thymus	T cell function (S.I.)		
	MLC	Mitogen Response	
		PHA	ConA
Normal	6.29 (12)	3.83 (17)	12.7 (17)
Abnormal	1.98 (16)[a]	4.39 (26)[b]	20.9 (26)[b]

[a] $p < 0.01$.
[b] Not significant.
S.I.: Stimulation Index; ( ): Number of mice.

Circulating immune complexes (CIC). The results of CIC using the Clq-SPRIA and Kg-SPRIA are shown in Table 4. The mean level of CIC in the group with abnormal thymus was higher than that in the group with normal thymus. A significant correlation between the titer of CIC and the thymic abnormalities was observed in Clq-SPRIA, but not in Kg-SPRIA.

Table 4. Relationship between circulating immune complexes and thymic abnormalities

Mice	Age (M)	Thymus	C.I.C.[a]	
			Clq-SPRIA	Kg-SPRIA
MRL/l	1-2	Normal (16)	3.70 ± 2.79	56.48 ± 20.78
		Abnormal (30)	9.59 ± 7.68[b]	67.16 ± 25.90
BALB/c	1-2	Normal (20)	0.58 ± 0.56	1.37 ± 0.97

[a] Agg MGG μg eq/ml.
[b] $P < 0.01$.
( ) Number of mice.

Thymus graft. We designed an experiment to attempt to examine thymic function using thymus grafts. Thymus from newborn MRL/l mice or MRL/l mice at 3 mo of age was grafted under the kidney capsule of BALB/c nu/nu mice. The results are summarized in Figure 4. Nude mice engrafted with newborn MRL/l thymus showed a remarkable responsiveness to Con A, MLC and anti-SRBC, whereas the nude mice engrafted with the thymus of 3 mo old MRL/l thymus grafts, grafts of thymus from 3 mo old autoimmune-resistant mice (BALB/c or C3H/He mice) regularly induced T cell functions in nude MRL/l thymus drafts, grafts of thymus from 3 mo old autoimmune-resistant mice (BALB/c or C3H/He) mice regularly induced T cell functions in nude mice. This finding indicates that the thymus of newborn MRL/l mice has the capacity to induce T cell functions, whereas thymic functions of MRL/l mice have already been largely lost by this age. This reduction of thymic functions is accompanied by the morphological thymic abnormalities described above.

Discussion

It has been reported that NZB and NZB/W F1 mice show thymic abnormalities, including premature degeneration of thymic epithelial cells and development of follicles within the thymic medullae [9, 11, 25]. Gershwin *et al.* [26] by *in vitro* culture analysis reported that thymic epithelial cells of NZB mice appear to lose their functions early as compared to those of other

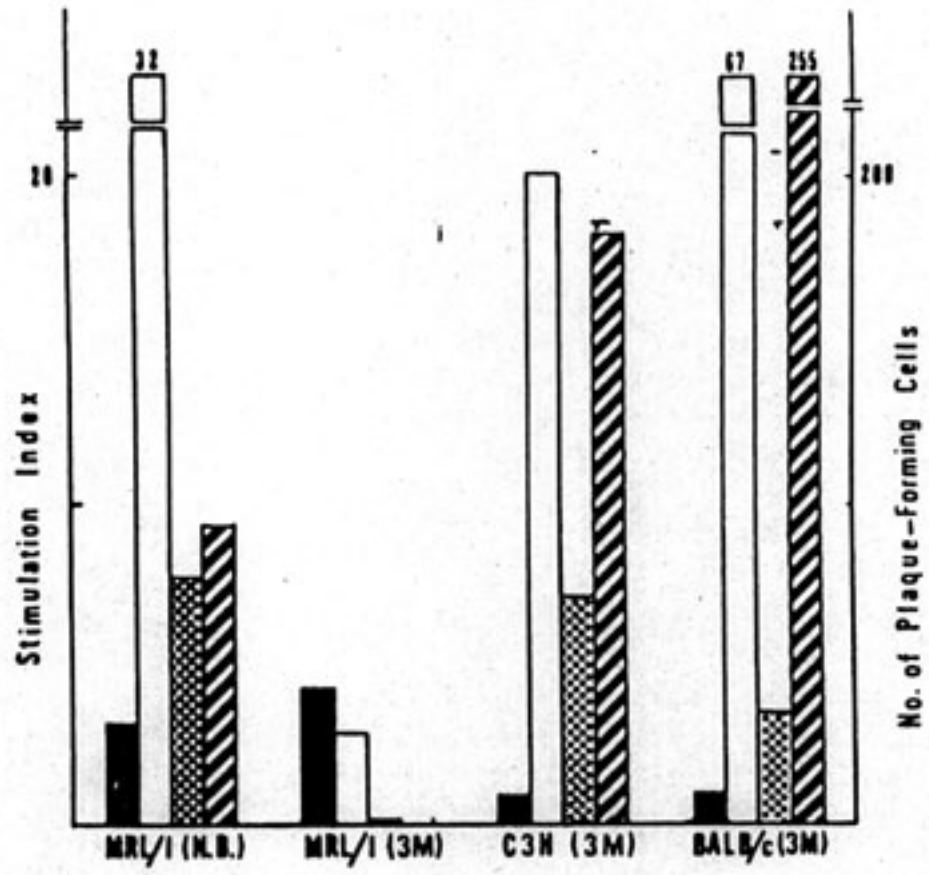

Figure 4. Induction of T cell functions in BALB/c nu/nu mice engrafted with the thymus of newborn MRL/l mice, 3 mo old MRL/l mice, 3 mo old C3H mice and 3 mo old BALB/c mice: Thy-1$^+$ cells ( ), Con A response ( ), MLC response ( ), PFC response ( ).

strains. Serum thymic factor (STF) levels have also been reported to decrease early in life in NZB [27].

In the present study, we found that MRL/l mice as well as NZB/W F1 mice show thymic abnormalities, including the infiltration of plasma cells and B lymphocytes which are not found in the normal thymus. In addition, these morphological abnormalities of the thymus preceded morphologic changes which occur in other organ sites where pathology regularly occurs in mice of the autoimmune-prone strains. Furthermore, the morphological abnormalities of the other organs were more remarkable in the group of mice with abnormal thymus than those in the group with normal thymus. In MRL/l mice it should be noted that the infiltration of plasma cells and B cells into the salivary gland was observed in 75% by the age of 3 mo. It has been reported that NZB and NZB/W F1 mice might be animal models for Sjogren syndrome, which appears with autoimmune sialoadenitis [28]. Judging from the earlier onset of these lesions and higher incidence, it seems likely that MRL/l mice provide even a better model for Sjogren syndrome than NZB and NZB/W F1 mice.

The group with abnormal thymus had a higher percentage of Thy-1$^+$ cells in the spleen than the group with normal thymus. However, these T cells did not show appropriate responsiveness to T cell mitogens. These results are consistent with a previous report [29] that spleen cells from MRL/l mice (older than 4 mo) responded poorly to Con A or allogeneic stimulator cells. Therefore, these observations reflect the proliferation of abnormal T cells which have lost or failed to develop their normal functions. Production of these abnormal T cells may result from abnormalities of the thymus which is a major central organ of differentiation of T cells [30]. It has been shown that deposits of Ig·and C (and sometimes DNA and gp70 retroviral envelope antigen) are found in the glomerulus of MRL/l and NZB/W F1 mice. These mice develop IC-type glomerulonephritis and die of uremia, and the development and progression of the renal disease is correlated with deposition of the anti gp70 complexes [31]. Several groups have reported that MRL/l mice show with age an elevation of CIC in sera [32–34]. In the present study using c1q-SPRIA, the high level of CIC in the mice with abnormal thymus was observed, as compared to those with normal thymus. In the assay of Kg-SPRIA, however, no difference between the group with an abnormal thymus and the normal group was found. It is possible that these differences result from the character of the CIC such as ability for complement fixation, and further studies using an assay method which is not dependent on complement may be indicated [35]. In the thymus graft experiments, we have found that the thymus of newborn MRL/l mice has the capacity to induce T cell functions in nude mice, whereas the thymus of 3 mo old mice does not have this capacity. Therefore, it is likely that the thymus of MRL/l mice shows both morphological and functional abnormalities with age. Kysda *et al.* [36] demonstrated that the transplantation of

thymus grafts from young syngeneic donors leads to increased survival of NZB/W F1 mice. Thus, it seems that deficiency of thymic immunoregulatory functions represents one major feature in the development of autoimmune diseases. However, it remains unclear why these mice show the early morphological and functional abnormalities of the thymus. Whether these thymic abnormalities are genetically determined or are secondary to other events is now under investigation.

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