Hiroshima J. Med. Sci.
Vol.45, No.3, 75～83, September, 1996
HIJM 45–12

Dysfunction of Immune System and Induction of Autoantibodies to Liver Antigens by Neonatal Thymectomy in Mice

Mitsuru MYOZAKI, Mei Qi WANG, Yasuhiro YOSHIDA, Jia Nuo LIU, Etsushi KURODA and Uki YAMASHITA

Department of Immunology and Parasitology, Hiroshima University School of Medicine, Hiroshima, Japan

ABSTRACT

We examined the development of autoantibodies to liver proteins and hepatitis in BALB/c mice thymectomized 2 days after birth and attempted to characterize the immune function of these mice. Autoantibodies to crude liver proteins detected by ELISA were found in 21(84%) of 25 mice thymectomized 2 days after birth. In these mice, sera of 11 animals showed reactivity with both liver specific proteins (LSP) and the second fraction of crude liver proteins; sera of 3 mice showed reactivity with only the second fraction but no sera showed reactivity with only LSP. By Western-immunoblotting, sera of BALB/c mice which showed high autoantibody level to liver proteins detected a strong band around 150kD in the second fraction of crude liver proteins. Still more, hepatic inflammation; mononuclear cell infiltration in the portal area, was induced in mice with apparently high autoantibody level to crude liver proteins. These results in BALB/c mice corresponded with our previous reports which employed C3H/HeN mice.

Next, we examined immune functions of mice thymectomized 2 days after birth. In thymectomized mice, the proportion of Thy-1, L3T4 and Lyt-2 positive cells (T cells) decreased and the proportion of B220 positive cells (B cells) increased. The proliferative response of lymph node lymphocytes cultured with mitomycin C-treated syngeneic spleen cells was lower, and the total IgG level in the sera was higher when compared with control normal mice. Anti-nuclear antibody (ANA) also appeared in the sera of thymectomized mice 2 days after birth.

All these results suggest that the dysfunction of T cell and polyclonal activation of B cell were induced in neonatally thymectomized mice and resulted in the production of ANA and autoantibodies to liver proteins.

Key words: *Neonatal thymectomy, Immune dysfunction, Autoantibody to liver*

The thymectomy of some strains of mice during the neonatal period, 2～4 days after birth, has been reported to induce some organ specific autoimmune diseases such as thyroiditis[9], oophoritis[16,25], gastritis[22,29], prostatitis[26] and orchitis without any additional sensitization. The dysfunction of T cells which regulate immune system has been considered the principal cause of these autoimmune diseases. The organ and the incidence of autoimmune diseases induced by neonatal thymectomy are various in strains of mice. There is one report of hepatitis induced spontaneously in nonwasting thymectomized mice[30]. However, it is widely accepted that hepatitis is not induced by neonatal thymectomy alone.

We previously reported that C3H/HeN mice thymectomized 2 days after birth produced autoantibodies reactive with crude liver proteins at a high rate. Hepatitis was induced in mice with an apparently high autoantibody level to crude liver proteins; we noted that autoantibodies, developed by neonatal thymectomy, reacted with liver specific antigen (LSP) and new pathogenic 150kD liver proteins other than LSP which have been not reported so far[15]. In order to confirm these results, we attempted to perform similar experiments using a different strain of mice and to examine the immune functions of mice thymectomized 2 days after birth.

MATERIALS AND METHODS

Animals

BALB/c(H-2^d) mice were obtained from Seiwa

Send all correspondence to : Dr. U. Yamashita, Department of Immunology and Parasitology, Hiroshima University, School of Medicine, 1–2–3 Kasumi, Minami-ku, Hiroshima 734, Japan

Experimental Animal Co. (Yoshitomi, Ohita, Japan) and maintained on purina rodent chow and water *ad libitum* in our laboratory.

Neonatal thymectomy

At 2 days after birth, thymectomy was carried out under ether anaesthesia with the aid of a dissecting microscope. After cutting the skin and the sternum of the mice, the thymus was removed by ablation with a swab and the wound was sutured. When the mice were sacrificed, the absence of thymus was confirmed. Control mice were sham-operated.

Preparation of stimulator spleen cells

Spleens were obtained from normal mice and spleen cell suspension was prepared after the treatment with red blood cell lysing buffer. Normal spleen cells (10×10^6/ml) were treated with 50 μg/ml mitomycin C (Kyowahakko Kogyo Co., Tokyo, Japan) for 1 hr at 37°C, washed four times, and then mixed with responder cells.

Autologus mixed lymphocyte reaction (AMLR)

Lymph-nodes (LN) were obtained from normal and neonatally thymectomized mice and lymphocytes were prepared as responder cells. Lymphocytes from LN (4×10^5) were mixed with mitomycin C-treated spleen cells (2×10^5) in 0.2ml of Eagle Hanks Amino Acid medium containing 10% fetal calf serum (FCS) in wells of flat-bottomed microtiter culture plates (#3072, Falcon Plastics, Oxnard, CA). They were cultured at 37°C in 5% CO_2 for 5, 7 and 9 days, labeled during the last 18 hours with 0.5 μCi of tritiated thymidine ([^{3}H]-TdR, Amersham International Plc., Buckinghamshire, UK) and were harvested with the aid of an automated cell harvester (Abe Kagaku Co., Chiba, Japan). The amount of radioactivity incorporated into DNA in the lymphocytes was measured with a liquid scintillation counter (LSC-3100, Aloka Co., Tokyo, Japan)[4]. The results were expressed as the mean counts per minute (cpm) of triplicate cultures with standard error.

Cell analysis by flow cytometry

Surface phenotype of lymphocytes from LN was identified by flow cytometry using monoclonal antibodies (mAb). 1×10^6 lymphocytes were treated with fluorescein isothiocyanate (FITC)- conjugated anti-Thy-1, anti-Lyt-2 mAb (Becton Dickinson Immunocytometry System, Mountain View, CA), anti-B220 mAb (Coulter Immunology, Hialeah, FL) and phycoerythrin (PE)- conjugated anti-L3T4 mAb (Becton Dickinson). The fluorescence-positive cells were analyzed by an EPICS Elite (Coulter).

Measurement of total IgG in sera

For the measurement of total IgG in sera, we used enzymed-linked solid phase immunosorbent assay (ELISA). Microtiter plates (Sumitomo Bakelite Co., Tokyo, Japan) were coated with anti-mouse IgG Ab (The Binding Site Ltd., Birmingham, UK) at a concentration of 5 μg/ml in Tris-EDTA-HCl buffer by incubating at 4°C for 20 hr. Then, the plates were washed four times with phosphate buffered saline, pH7.2 (PBS) – Tween20 (0.05%). Mice sera were diluted to 10000× with 1% bovine serum albumin (BSA, Sigma Chemical Co., St.Louis, MO)-PBS-Tween 20. This optimum dilution was pre-established by titrating the sera from 100× to 100000×. 100 μl of the diluted sera was added in duplicate to the coated wells and subsequently incubated at 4°C for 3 hr. The plates were then washed four times with PBS-Tween 20 and 100 μl of alkaline phosphatase-conjugated F(ab')$_2$ fraction of goat anti-mouse IgG antibody (Tago Inc., Burlingame, CA) diluted 2100× with BSA-PBS-Tween 20 was added to each well and incubated at room temperature for 3.5 hr. The plates were then washed three times with PBS-Tween 20 and once with distilled water. For the enzyme reaction, 100 μl of substrate, p-nitrophenylphosphate (Zymed Laboratory Inc., San Francisco, CA) was added and incubated at 37°C for 30 min. The reaction was stopped with 100 μl of 0.15M NaOH and the colour intensity was measured by an absorption at 405nm of the wave length by Immuno Reader (NJ-2000, Nippon Intermed Co., Tokyo, Japan). Purified mouse IgG was used as a control.

Preparation of crude liver proteins

The liver of normal mice was canulated and perfused several times with PBS. The liver was then cut into pieces and suspended in a double volume of 0.25 M Sucrose(adjusted to pH 8.0 with 0.1 N NaOH, Sigma Chemical Co.), and the mixture was homogenized using a Potter homogenizer. The homogenate was centrifuged at 105,000g for 60 min at 4°C. The supernatants obtained were denoted crude liver proteins.

Gel filtration of crude liver proteins

According to the method of McFarlane et al[13], crude liver proteins were fractionated on a Sepharose 6B column (3.5×64 cm, 540ml volume, Pharmacia Biochemical Co., Uppsala, Sweden) equilibrated with a buffer containing 0.1M Tris HCl, 0.2M NaCl and 1mM disodium ethylenediamine tetraacetate (EDTA) (pH8.0). The sample was eluted at 17ml/hr with the buffer and each 6 ml fraction was collected. An absorption at 280nm of each fraction was measured by photometer (Ubest V-520, JASCO Co., Tokyo, Japan)

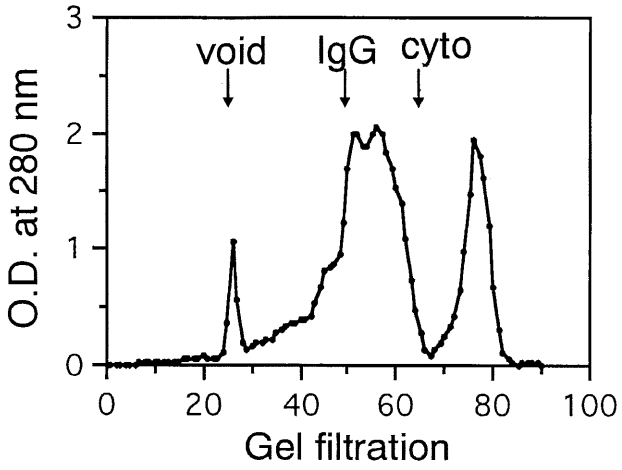


Fig. 1. Fractionation of crude liver proteins by Sepharose 6B column. Crude liver proteins were fractionated by a Sepharose 6B column (540ml) and each 6ml fraction was collected. The protein concentration was detected by O.D. at 280nm. The abscissa is the fraction number. The column size was calibrated by blue dextran (void volume), human immunoglobulin G (IgG, 150kD) and cytochrome *c* (Cyto, 12kD).

(Fig. 1). The size of the column was calibrated by eluting blue dextran (void volume), human immunoglobulin G (IgG, 150kD) and cytochrome *c* (12kD, Sigma Chemical Co.). The protein peak eluted in the void volume was collected and used as LSP. LSP was previously reported as the principle target antigen of liver. The second peak was collected and was denoted the second fraction of crude liver proteins. The protein concentrations of LSP and the second fraction were measured according to the method of Lowry et al[12].

Detection of autoantibody to crude liver proteins, LSP and the second fraction

For the detection of autoantibody to crude liver proteins, LSP and the second fraction, we used ELISA. The method was almost the same as that used to detect total IgG. Microtiter plates were coated with each antigen at a concentration of 50 μg/ml and mice sera were diluted to 100×. This optimum dilution was pre-established by titrating the sera from 20× to 2560×. Still more, we used peroxidase-conjugated goat anti-mouse IgG antibody (The Binding Site Ltd.), diluted 400× with BSA-PBS-Tween 20 as the second antibody and *o*-phenylenediamine (Sigma Chemical Co.) for the enzyme reaction. The reaction was stopped with 8N H_2SO_4 and the colour intensity was measured at 490nm. The antibody level was expressed as the specific optical density in which the optical density of wells with antigen alone and serum alone was subtracted from the optical density of wells of experimental group.

Detection of anti-nuclear antibodies (ANA)

For the detection of ANA, we used immunohistochemical examination. The smeared HEp-2 cells on the slides were treated with sera diluted from 10× to 640× at 37°C for 30 min in a humidified chamber. After washing by PBS, they were incubated with 20× diluted peroxidase-conjugated sheep anti-mouse IgG antibody at 37°C for 30 min. After washing, they were stained with 3, 3-diaminobenzidine (DAB, Kyowa Medix Co., Tokyo, Japan) for 5 min to develop the colour and washed by water. Subsquently, we observed the stain of nuclei of HEp-2 cells microscopically.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

For the separation of crude liver proteins, SDS-PAGE was performed using 10% polyacryl amide gel (MULTIGEL 10, Daiichi Pure Chemicals Co., Tokyo, Japan) according to the modified method of Laemmli[10]. Samples adjusted to approximately 1mg/ml were dissolved in sample buffer (0.0625M Tris-HCl, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, pH6.8, Sigma Chemical Co.) and boiled for 10 min. The samples were applied on gel and electrophoresed at 40mA for about 1 hr. After completion of the separation, the contents of the gels were electroblotted over to nitrocellulose paper (Trans-Blot, Bio-Rad Laboratories, Hercules, CA) at 5V for 75 minutes[28]. The nitrocellulose strips were stained with colloidal gold stain for protein blots (AuroDye Forte kit, Amersham Plc.) or immunoblotted as follows. The nitrocellulose strips were incubated with 2% BSA-PBS at 4°C for 20 hr for blocking. After washing, mice sera diluted to 200× with 0.2% BSA-PBS-Tween 20 were added and incubated at 4°C for 20 hr. This optimum dilution was pre-established by titrating the serum from 10× to 2000×. The nitrocellulose strips were then again washed and alkaline phosphatase-conjugated $F(ab')_2$ fraction of goat anti-mouse IgG antibody (The Binding Site Ltd.), diluted 1000× with 0.2% BSA-PBS-Tween20, was added and incubated at room temperature for 3 hr. For the enzyme reaction, alkaline phosphatase substrate KIT II (Vector Laboratories Inc., Burlingame, CA) was used in 100mM Tris-HCl buffer, pH9.5.

Histological examination of liver tissue

Liver specimens were fixed with 10% buffered formalin, cut and stained with hematoxylin-eosin. Sections were microscopically examined.

Assay for plasma transaminase activities

Plasma transaminase activity, glutamic-pyruvic transaminase (GPT), was measured by the UV method using GPT opt kit (Boehringer Mannheim, Mannheim, Germany).

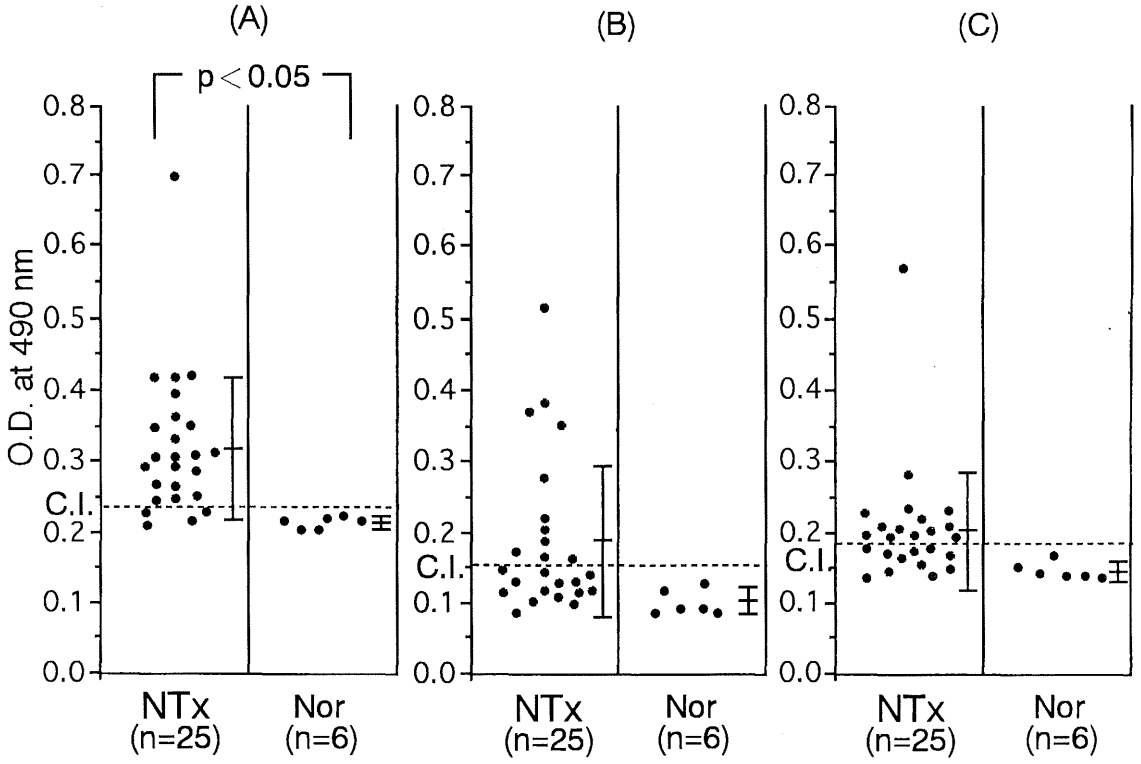


Fig. 2. Incidence of autoantibody to liver proteins in normal (Nor) and thymectomized (NTx) mice. BALB/c mice were thymectomized 2 days after birth. At 8 weeks sera were collected and the reactivity with liver proteins was detected by ELISA. Results are shown by O.D. at 490nm. (A) reactivity with crude liver proteins. (B) reactivity with LSP. (C) reactivity with the second fraction. C.I.=cut off index whose value is mean + 3 standard deviations of O.D. of normal sera.

Experimental protocol

Mice were classified into 2 groups; mice thymectomized 2 days after birth and sham-operated normal mice as control. Thymectomized mice and sham-operated normal mice were raised in cages side by side during the same period in order to adjust environment as similar as possible. Blood was collected from the retroorbital plexus 8 weeks after birth to examine antibody activities. At 12 weeks, the mice were sacrificed and the livers were removed for histological examination.

Statistics

Statistical analysis was performed by Student's t-test. A confidence level of <0.05 was considered significnt[31].

RESULTS

Induction of autoantibody to liver proteins by neonatal thymectomy in mice

BALB/c mice were thymectomized 2 days after birth and the reactivity of their sera and control sera against crude liver proteins, LSP and the second fraction were investigated 8 weeks after birth. As shown in Fig. 2A, the antibody level to crude liver proteins, measured as optical density (O.D.), was significantly different between 25 mice thymectomized 2 days after birth and 6 control mice ($p<0.05$). The mean value of the control group was 0.216 ± 0.008 (mean $\pm$ s.d.) and that of thymectomized mice 2 days after birth was 0.321 ± 0.102. If a lower limit value for positivity was set at O.D.=0.240 which was the control mean plus three standard deviations, 21 of 25 thymectomized mice (84%) were positive. Regarding the antibody level to LSP and the second fraction, 11 (44%) and 14 (56%) of 25 thymectomized mice were positive, respectively (Fig. 2B, 2C). In these experiments, O.D. values of wells of antigen alone and serum alone were subtracted. Accordingly, this difference was not caused by a non specific binding of sera from thymectomized mice on the plates. We examined the relation between autoantibodies to LSP and autoantibodies to the second fraction. As shown in Fig. 3, the sera of 11 mice showed positive levels to both LSP and the second fraction, the sera of 3 mice showed a positive level to only the second fraction, and none sera showed positive level to only LSP.

Separation of the second fraction of crude liver proteins by SDS-PAGE and immunoblotting

Next, we separated crude liver proteins by SDS-PAGE and detected by immunoblotting. The sepa-

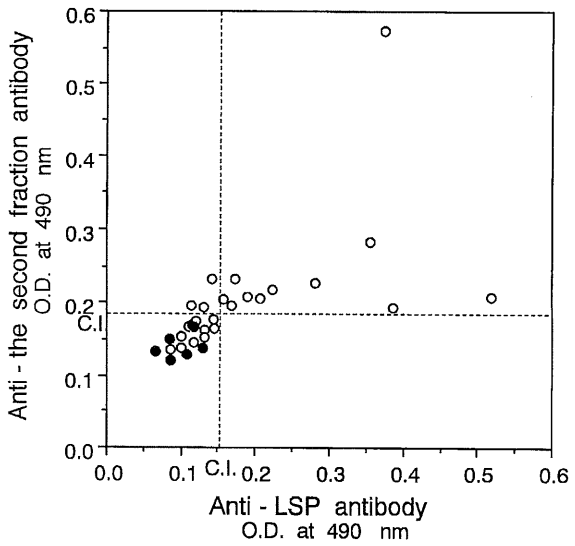


Fig. 3. The relation between the reactivity with LSP and the second fraction.
The sera from normal (closed circle) and thymectomized BALB/c mice (open circle) in Fig. 2. were examined using the second fraction (ordinate) and LSP (abscissa). C.I.=cut off index whose value is mean + 3 standard deviations of O.D. of normal sera.

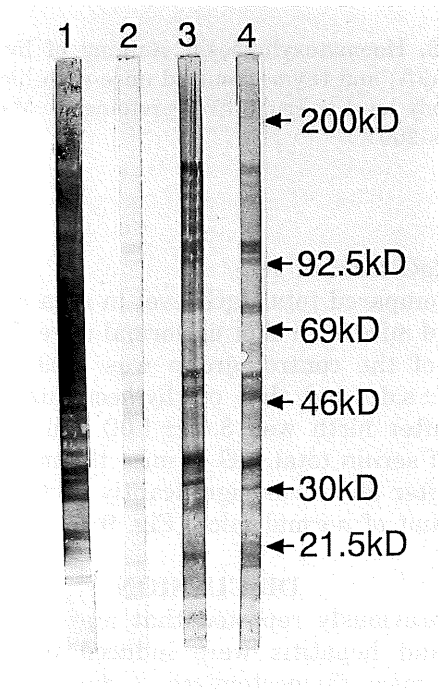


Fig. 4. Immunoblotting of crude liver proteins.
Crude liver proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and immunostained. Column 1, protein staining ; 2, immunostained by normal mouse serum ; 3,4, immunostained by serum from thymectomized mice in Fig.3 with high reactivities to the second fraction.

ration of crude liver proteins by SDS-PAGE showed that the preparation contained a complex mixture of proteins (Fig. 4-1). Fig. 4–2 showed the reactivity of sera from normal mouse. The sera of thymectomized BALB/c mice which showed a high reactivity with the second fraction of crude liver proteins by ELISA detected a strong band around 150kD and some weak bands between 150kD and 100kD (Fig. 4–3 and –4).

Anti-nuclear antibody (ANA)

ANA was detected with the smeared HEp-2 cells on slides by the standard immunohistochemical analysis. We observed them with a microscope of 200× magnifications and judged as follows: the serum with clear staining of nuclei is (+), the serum with slight staining of nuclei is (±), the serum without staining of nuclei is (–) for ANA. ANA was (–) in sera of all normal mice at 20× dilution (Fig. 5A). 4 thymectomized mice showed (+) at 160× dilution (Fig. 5B) and (±) at 320× dilution. The other 4 thymectomized mice showed (+) at 80× dilution and (±) at 160× dilution. Another 13 thymectomized mice showed (±) at 40× dilution. Thus, we confirmed that ANA appeared in sera of thymectomized mice 2 days after birth.

Histological examination of the liver

Hepatic inflammation; mononuclear cell infiltration in the portal area (Fig. 6B) was seen in thymectomized mice with high autoantibody levels to crude liver proteins. However, necrosis of hepatocytes was not clearly observed in the parenchyma. Histological change was not seen in thymectomized mice with low autoantibody levels to crude liver proteins and control normal mice (Fig. 6A).

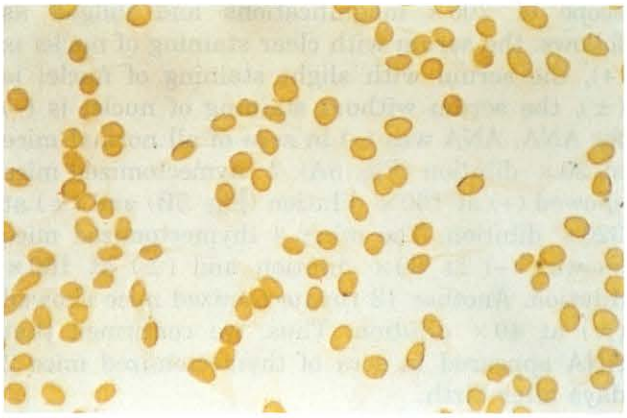
Plasma transaminase activities

Glutamic-pyruvic transaminase (GPT) in the blood, an indicator of the injury of hepatocytes, was not elevated in the thymectomized mice with hepatitis compared with the thymectomized and normal mice without hepatitis (Table 1).

Table 1. Serum GPT levels in normal (Nor) and thymectomized (NTx) mice

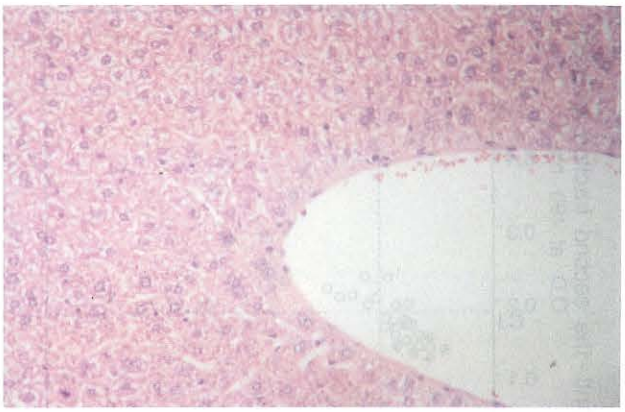
mice	No. of mice	GPT
		IU/liter
NTx with hepatitis	4	12.0±17.9
NTx without hepatitis	7	11.4±10.7
Nor	10	20.0±13.3

5A

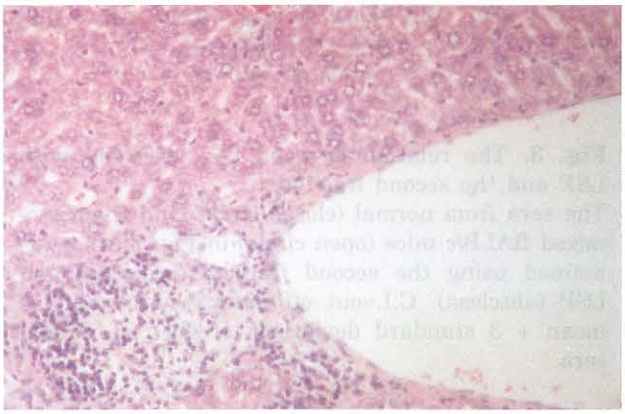


5B

Fig. 5. Anti-nuclear antibodies (ANA) were detected with sections of HEp-2 cells by immunohistochemical staining.
(A): The section of HEp-2 cells was treated with 20× diluted serum from normal mouse, followed by peroxidase-labeled sheep anti-mouse IgG and DAB. ANA is negative.
(B): The section was treated with 160× diluted serum from thymectomized mouse. ANA is positive. Magnification is 200×.



6A



6B

Fig. 6. Haematoxylin-eosin staining of liver from normal(A) and thymectomized mice with high autoantibody level to crude liver proteins(B). Magnification is 200×.

Surface phenotype of lymphocytes from LN

Next, we studied the immunological functions of neonatally thymectomized mice. The proportion of Thy-1, L3T4 and Lyt-2 positive cells (T cells) decreased and the proportion of B220 positive cells (B cells) increased in thymectomized mice as compared with normal mice. Still more, the proportion of cells lacking both Thy-1 and B220 increased in thymectomized mice (Fig. 7).

Autologus mixed lymphocyte reaction (AMLR)

The *in vitro* culture of LN lymphocytes with mitomycin C-treated syngeneic spleen cells induced the proliferative responses (AMLR). However, the AMLR of thymectomized mice was significantly lower than that of nomal mice ($p<0.05$). The proliferative responses increased slowly on days 5–9 in normal mice but not in thymectomized mice (Fig. 8).

Total IgG level in sera

We compared total IgG level in sera of thymectomized mice with that of normal mice. The mean value of the control group was 2.29 ± 0.61 g/dl (mean ± s.d.) and that of thymectomized mice 2 days after birth was 3.45 ± 1.02 g/dl. Thus, the level of serum total IgG of mice thymectomized 2 days after birth was significantly ($p<0.05$) higher than that of normal mice (Fig. 9).

DISCUSSION

We previously reported that autoantibodies to liver and hepatitis were induced in C3H/HeN ($H\text{-}2^k$) mice thymectomized 2 days after birth, and that autoantibodies induced by neonatal thymectomy reacted with LSP and new pathogenic 150kD liver proteins other than LSP[15]. To further confirm this point, we performed similar experiments using different strain of mice, BALB/c ($H\text{-}2^d$).

In this study, we demonstrated that 84% of BALB/c mice thymectomized 2 days after birth produced autoantibody to crude liver proteins. In

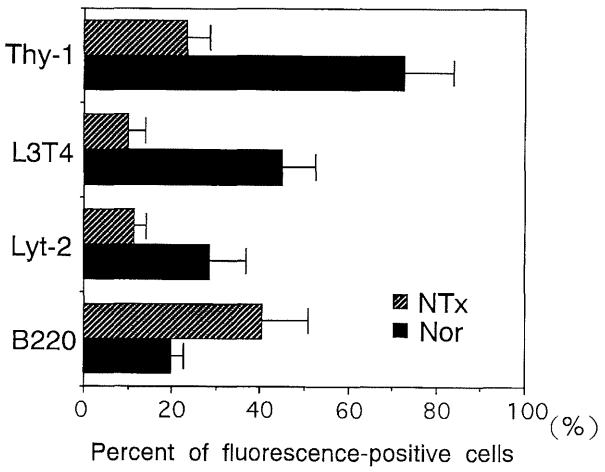


Fig. 7. Surface marker of lymph node lymphocytes from normal (Nor) and thymectomized (NTx) mice. Lymph-node lymphocytes were stained with FITC-anti-Thy-1, Lyt-2, B220 and PE-anti-L3T4 antibodies and the fluorescence-positive cells were analyzed by flow cytometry.
The results show the percentage of antibody-positive cells (mean ± s.d.) of 5 mice.

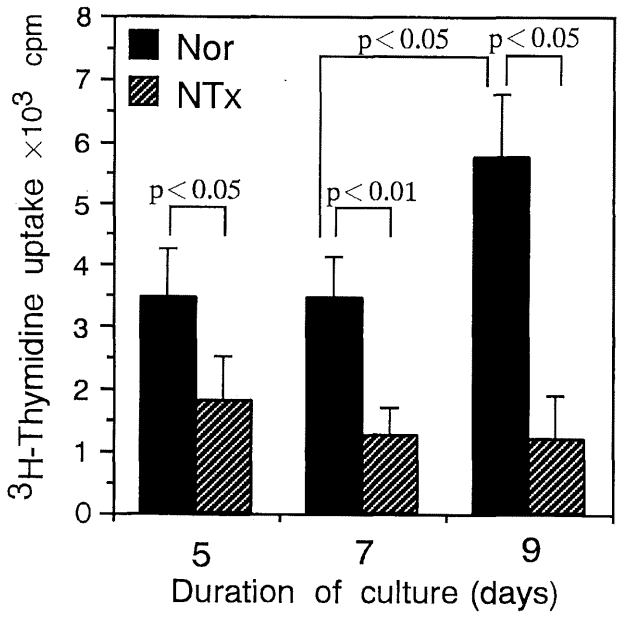


Fig. 8. Autologus mixed lymphocyte reaction.
4×10^5 lymph-node lymphocytes (responder cells) from normal (Nor) or thymectomized (NTx) mice and 2×10^5 mitomycin C-treated splenocytes from normal mice (stimulator cells) were mixed together, cultured for 5, 7 and 9 days, pulsed with 0.5 μCi/well of [^{3}H]-TdR for the last 18 h and harvested. [^{3}H]-TdR incorporated by lymphocytes was counted by a liquid scintillation counter. The results show the cpm (mean ± s.d.) of 5 mice.

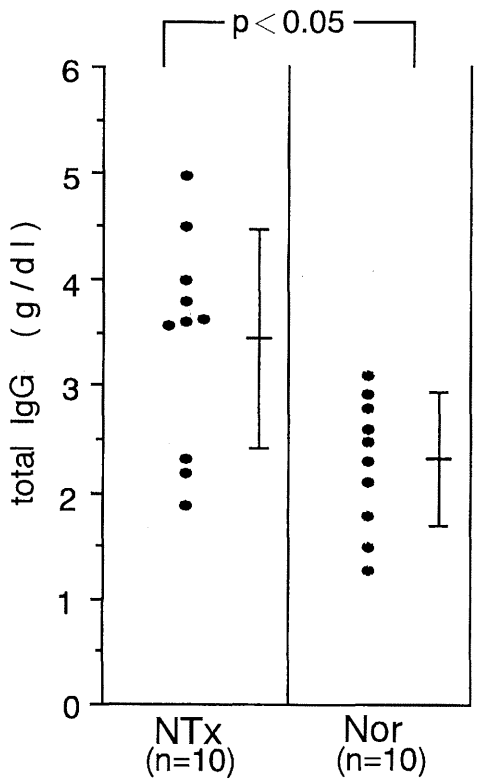


Fig.9. Total IgG levels in sera from normal (Nor) and thymectomized (NTx) mice.
At 8 weeks sera were collected and the level of total IgG in sera was detected by ELISA.

thymectomized mice which show positive for autoantibody to crude liver proteins, 52% were positive for both LSP and the second fraction, 14% were positive for only the second fraction; none were positive for only LSP. In Western-immunoblotting, the principle target antigens in the second fraction were regarded as 150kD proteins (major band) and some other proteins between 150kD and 100kD (minor bands). These results correspond with our previous report using C3H/HeN mice and suggest that the induction of autoantibodies to liver proteins by neonatal thymectomy is a general phenomenon. In order to discuss the genetic control of the induction of liver-specific autoantibodies, we have to further study some other strains. The evidence reported here indicates to us the possibility of preparing liver-specific monoclonal autoantibody, because almost all parent cells for hybridoma are derived from BALB/c mice. The sera of thymectomized mice also contained autoantibodies to other crude proteins, such as kidney, lung, stomach and brain (data not shown) and ANA detected with smeared HEp-2 cells. Next, we attempted to examine immune functions of mice thymectomized 2 days after birth. In the LN of thymectomized mice, the proportion of Thy-1, L3T4 and Lyt-2 positive cells (T cells) decreased and the proportion of B220 positive cells (B cells) increased. The proliferative response of lymphocytes from LN against mitomycin C-treated syngeneic spleen cells (AMLR)

was significantly lower than that of control mice, and the delayed response observed in normal mice was not detected in thymectomized mice. These results indicate that not only the decrease in the proportion of T cells but also the difference of T cell response to autoantigen exists in thymectomized mice. It is reported that suppressor T cells present in neonatal mice are non-specific and short-lived; the development of these T cells requires the presence of thymus for at least 7 days after birth[8], and specific suppressor T cells develop by recognizing autoantigens[19] and migrate to periphery. Thus, they are considered to be activated by specific self antigens from each peripheral organ to maintain tolerance[27]. Therefore, suppressor T cells in mice thymectomized 2 days after birth may be not sufficiently educated. Some studies have reported that responder cells in AMLR had characteristics of suppressor T cells[18] and the AMLR was defective in patients with autoimmune diseases[6,14] and several inbred strains of mice genetically susceptible to autoimmune diseases[5,23]. Therefore, we suspect that the lower proliferative response in AMLR reflects the decrease and dysfunction of suppressor T cells maintaining tolerance in thymectomized mice. Smith and Talal reported that the deficient AMLR was a state of the decreased suppressor T cell activity to regulate autoreactive lymphocyte clones and the failure to remove modified self[24]. On the other hand, the level of serum total IgG is higher in mice thymectomized 2 days after birth than in normal mice. We consider that B cells are activated polyclonally because of the decrease and the dysfunction of suppressor T cells in neonatally thymectomized mice.

It is widely accepted that hepatitis is rarely induced by neonatal thymectomy alone, but hepatic inflammation was seen in thymectomized BALB/c mice with high autoantibody levels to crude liver proteins. This result corresponds with our previous report using C3H/HeN mice. Recently, it is suspected that double negative T cells are related to autoimmune diseases[3,21] and extrathymic T cell lineages; moreover, the concept of T cell maturation in extrathymic sites such as intestine[11], peritoneal cavity[2] and liver has been generally accepted. In particular, the liver is reported to be the major hematopoietic organ in the fetal stage[7] and an important organ for the production of a unique T cell population including forbidden clones[1,17,20]. By neonatal thymectomy, the extrathymic T cell maturation is suspected to be activated most remarkably in the liver. In a previous report, we demonstrated that infiltrating T cells in portal area of liver belonged to L3T4(–)Lyt-2(–) (double negative) cell population[15].

We conclude that neonatal thymectomy results in the decrease and dysfunction of suppressor T cells and the activation of extrathymic T cell lineages reactive with autoantigens, and polyclonal B cell activation is induced. Therefore, we consider that various autoantibodies, including autoantibody to liver, are induced consequentially in neonatally thymectomized mice.

ACKNOWLEDGEMENT

This work was supported in part from a grant-in-aid of Fukuyama Transporting Shibuya Longevity and Health Foundation.

(Received April 8, 1996)
(Accepted July 16, 1996)

REFERENCES

1. **Abo, T., Ohteki, T., Seki, S., Koyamada, N., Yoshikai, Y., Masuda, T., Rikiishi, H. and Kumagai, K.** 1991. The appearance of T cells bearing self-reactive T cell receptor in the livers of mice injected with bacteria. J. Exp. Med. **174:** 417–424.
2. **Andreu-Sanchez, J.L., De Alboran, I.M., Marcos, M.A.R., Sanchez-Movilla, A., Martinez, A.C. and Kroemer, G.** 1991. Interleukin 2 abrogates the nonresponsive state of T cells expressing a forbidden T cell receptor repertoire and induces autoimmune disease in neonatally thymectomized mice. J. Exp. Med. **173:** 1323–1329.
3. **Davidson, W.F., Dumont, F.J., Bedigian, H.G., Fowlkes, B.J. and Morse, H.C. III.** 1986. Phenotypic, functional, and molecular genetic comparisons of the abnormal lymphoid cells of C3H-lpr/lpr and C3H-gld/gld mice. J. Immunol. **136:** 4075–4084.
4. **Harrison, M. R., Thurmond, G. B. and Thomas, G.M.** 1974. A simple and versatile harvesting device for processing radioactive label incorporated into and/or released from cells in microculture. J. Immunol. Methods **4:** 11–18.
5. **Hom, J.T. and Talal, N.** 1982. Decreased syngeneic mixed lymphocyte reaction in autoimmune susceptible mice. Scand. J. Immunol. **15:** 195–203.
6. **James, S.P., Elson, C.O., Waggoner, J.G., Jones, E.A. and Strober, W.** 1980. Deficiency of the autologous mixed lymphocyte reaction in patients with primary biliary cirrhosis. J. Clin. Invest. **66:** 1305–1310.
7. **Kamps, W.A., Timens, W., De Boer, G.J., Spanjer, H.H. and Poppema, S.** 1989. In situ study of haemopoiesis in human fetal liver. Scand. J. Immunol. **30:** 399–408.
8. **Kawauchi, H., Taniguchi, K., Kubo, C., Shimamoto, Y. and Nomoto, K.** 1983. The mechanism of reduction of cell-mediated cytotoxicity in neonatally thymectomized mice. Immunology **50:** 199–205.
9. **Kojima, A., Tanaka-Kojima, Y., Sakakura, T. and Nishizuka, Y.** 1976. Prevention of postthymectomy autoimmune thyroiditis in mice. Laboratory Investigation **34:** 601–605.
10. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of

bacteriophage T4. Nature **227:** 680–685.
11. **Lefrancois, L. and Olson, S.** 1994. A novel pathway of thymus directed T lymphocyte maturation. J. Immunol. **153:** 987–995.
12. **Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.** 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. **193:** 265–275.
13. **McFarlane, I.G., Wojcicka, B.M., Zucker, G.M., Eddleston, A.L.W.F. and Williams, R.** 1977. Purification and characterization of human liver-specific membrane lipoprotein (LSP). Clin. Exp. Immunol. **27:** 381–390.
14. **Miyasaka, N., Sauvezie, B., Pierce, D.A., Daniels, T.E. and Talal, N.** 1980. Decreased autologous mixed lymphocyte reaction in Sjogren's syndrome. J. Clin. Invest. **66:** 928–933.
15. **Myozaki, M., Kamiyasu, M., Miura, T., Watanabe, Y., Nakanishi, T. and Yamashita, U.** 1996. Induction of autoimmune hepatitis and autoantibodies to liver antigens by neonatal thymectomy in mice. Clin. Exp. Immuno. **104:** 133–143.
16. **Nishizuka, Y., Sakakura, T. and Tanaka, Y.** 1971. Ovarian dysgenesis induced by neonatal thymectomy in the mouse. Endocrinology **89:** 886–893.
17. **Ohteki, T., Seki, S., Abo, T. and Kumagai, K.** 1990. Liver is a possible site for the proliferation of abnormal CD3+4–8– double-negative lymphocytes in autoimmune MRL-lpr/lpr mice. J. Exp. Med. **172:** 7–12.
18. **Pasternak, R.D., Bocchieri, M.H. and Smith, J.B.** 1980. Surface phenotype of responder cells in the syngeneic mixed lymphocyte reaction in mice. Cellular Immunology **49:** 384–389.
19. **Sakaguchi, S., Takahashi, T. and Nishizuka, Y.** 1982. Study on cellular events in post-thymectomy auto-immune oophoritis in mice. J. Exp. Med. **156:** 1577–1586.
20. **Seki, S., Abo, T., Masuda, T., Ohteki, T., Kanno, A., Takeda, K., Rikiishi, H., Nagura, H. and Kumagai, K.** 1990. Identification of activated T cell recepter $\gamma\delta$ lymphocytes in the liver of tumor-bearing hosts. J. Clin. Invest. **86:** 409–415.
21. **Shivakumar, S., Tsokos, G.C. and Datta, S.K.** 1989. T cell receptor $\alpha\beta$ expressing double-negative (CD4–/CD8–) and CD4 T helper cells in humans augment the production of pathogenic anti-DNA autoantibodies associated with lupus nephritis. J. Immunol. **143:** 103–112.
22. **Smith, H., Lou, Y.H., Lacy, P. and Tung, K.S.K.** 1992. Tolerance mechanism in experimental ovarian and gastric autoimmune diseases. J. Immunol. **149:** 2212–2218.
23. **Smith, J.B. and Pasternak, R.D.** 1978. Syngeneic mixed lymphocyte reaction in mice: strain distribution, kinetics, participating cells, and absence in NZB mice. J. Immunol. **121:** 1889–1892.
24. **Smith, J.B. and Talal, N.** 1982. Significance of self-recognition and interleukin-2 for immuno-regulation, autoimmunity and cancer. Scand. J. Immunol. **16:** 269–278.
25. **Taguchi, O., Nishizuka, Y., Sakakura, T. and Kojima, A.** 1980. Autoimmune oophoritis in thymectomized mice : detection of circulating antibodies against oocytes. Clin. Exp. Immunol. **40:** 540–553.
26. **Taguchi, O., Kojima, A. and Nishizuka, Y.** 1985. Experimental autoimmune prostatitis after neonatal thymectomy in the mouse. Clin. Exp. Immunol. **60:** 123–129.
27. **Taguchi, O., Kontani, K., Ikeda, H., Kezuka, T., Takeuchi, M. and Takahashi, T.** 1994. Tissue-specific suppressor T cells involved in self-tolerance are activated extrathymically by self-antigens. Immunology **82:** 365–369.
28. **Towbin, H., Staehelin, T. and Gordon, J.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets : procedure and some applications. Proc. Natl. Acad. Sci. USA **76:** 4350–4354.
29. **Tung, K.S.K., Smith, S., Matzner, P., Kasai, K., Oliver, J., Feuchter, F. and Anderson, R.E.** 1987. Murine autoimmune oophoritis, epididymoorchitis and gastritis induced by day 3 thymectomy. Am. J. Pathol. **126:** 303–314.
30. **Yunis, E.J., Teague, P.O., Stutman, O. and Good, R.A.** 1969. Post-thymectomy autoimmune phenomena in mice. Laboratory Investigation **20:** 46–61.
31. **Zan, J.H.** 1974. Biostatistical analysis. Prentice-Hall Inc., New Jergy.