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Effects of Splenectomy on Rat Polymorphonuclear Neutrophil Autoimmunity

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Summary. **Postsplenectomy effects on neutrophil autoimmune reactions were studied in 27 splenectomized Lewis rats. Either levamisole or lysozyme was injected into the splenectomized and non-splenectomized Lewis rats. In five of the 27 splenectomized rats, $CD8^+$ T cell numbers of mesenteric lymph nodes (MLN) decreased to less than 10%. $CD5^+$ cell proliferation of MLN was found in several rats. Activated B cells reacted to anti $CD5^+$ antibody. Decreased phagocytosis of polymorphonuclear (PMN) neutrophils was shown in both rats immunized with either levamisole or lysozyme. Anti-lysozyme antibody titer was generally increased in all 16 rats treated with lysozyme. On the peripheral blood smear of one splenectomized female with high anti-lysozyme antibody, 81% PMN neutrophils had a hypersegmented hyperchromatic nucleus of more than 7 lobes. Most of the hypersegmented PMN neutrophils lost their peroxidase reactions. The hepatocytes of the male immunized with lysozyme after splenectomy showed mitochondrial proliferation and many peroxisomes. Well developed rough endoplasmic reticulums were also recognized at the perinuclear area.**

This study indicated that splenectomy intensified the auto PMN neutrophil reactions when degranulation of myeloperoxidase had been occurred by anti-lysozyme antibody. Lysozyme in the spleen worked to maintain the stability of mitochondrial protein synthesis in the liver.

Key words—splenectomy, levamisole, lysozyme, $CD5^+$ cell, autoimmunity, PMN neutrophil.

INTRODUCTION

It is well known that severe postsplenectomy infections occur frequently. Even though O_2^- leakage from vacuoles was elevated in peritoneal macrophage, splenectomized mice macrophages showed a decrease in phagocytosis and killing.[1] A Kupffer cell-mediated decrease in hepatocyte protein synthesis has been seen until 60 days after splenectomy, when lipopolysaccharide was added to the cocultures.[2] On the other hand, perinuclear anti-neutrophil cytoplasmic autoantibodies (P-ANCA) to myeloperoxidase have been identified in many patients with systemic necrotizing vasculities.[3,4] Levamisole, which is used as an immuno-potentiating drug, has been reported to cause immune-mediated granulocytopenia.[5]

This paper studied polymorphonuclear (PMN) neutrophil immune dysfunctions inducted in rats injected with either levamisole or lysozyme after splenectomy. The effects of splenectomy on auto PMN neutrophil reactions were shown by a $CD8^+$ T cell decrease and $CD5^+$ B cell proliferation in mesenteric lymph nodes (MLN), the disturbed phagocytosis of PMN neutrophils, and hypersegmented hyperchromatic PMN neutrophils.

MATERIALS AND METHODS

Animals

Male specimens were Lewis rats ($RT1^l$) that were maintained in the colony of the Hamamatsu University School of Medicine. Females were LEW/Sea strain rats that were obtained from Seiwa Laboratory Animal Research Center (Fukuoka-ken, Japan). Experiment (Exp.) F and G F1 and Exp. A, B, C, D, E and F2 Lewis rats were used in this study. Splenectomy of Exp. A and B rats was done at 5 weeks of age. Splenectomy of Exp. C and D rats was done at 7 weeks of age. Exp. F rats were splenectomized at 20 weeks of age after three injections with

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levamisole (Sigma Chemical Co., U.S.A.). All these Lewis rats were kept and followed in controlled conditions.

Immunizations

Four splenectomized males of Exp. A were followed for 45 days after splenectomy. Six splenectomized females of Exp. B were followed for 48 days after splenectomy without any immunization. Chicken egg lysozyme-salt free (11,176 U/mg) was purchased from Washington Biochemical Corporation (U.S.A.). Emulsion of the lysozyme was made in Freund complete adjuvant (latron, Japan). Five splenectomized males of Exp. C were immunized subcutaneously with the lysozyme emulsion at concentrations of 1.2 mg, 2.6 mg, 2.8 mg and 4.0 mg per rat. Immunization began 21 days after splenectomy at 1-2 week intervals. They were observed for 28 days from the first immunization. Six splenectomized females of Exp. D were immunized subcutaneously with the lysozyme emulsion at concentrations of 1.1 mg, 2.6 mg, 2.0 mg and 1.0 mg per rat. Immunization began 21 days after splenectomy at 1-4 week intervals. They were observed for 43 days from the first immunization. Four non-splenectomized males of Exp. E were injected with the lysozyme emulsion at concentrations of 1.5 mg, 6.9 mg and 1.2 mg per rat subcutaneously, and with lysozyme suspension of 3.0 mg per rat intravenously. Immunization intervals were 1-4 weeks. They were followed for 49 days after the first immunization. The emulsion of levamisole was also made in Freund complete adjuvant. Six splenectomized Exp. F and 6 Exp. G females at 8 weeks of age were subcutaneously injected with the levamisole emulsion at concentrations of 1.6 mg, 2.6 mg, 3.0 mg, 4.4 mg and 2.0 mg per rat. Immunization intervals were 0.5-2 weeks. Both groups of the rats were followed for 135 days.

Passive cutaneous anaphylaxis (PCA) reaction

Anti DNP-Ascaris (LSL Co., Ltd., Japan) was diluted 120 times with saline. One hundred μl of anti DNP-Ascaris was subcutaneously injected into the back skins of 4 Exp. A males and four control males of 6 months of age. After 48 h, 0.9 ml of 0.5% Evans blue and 0.1 ml of 1 mg chicken egg lysozyme were injected into tail vein. A PCA reaction and a blue spot within 30 min were observed in the rats. All the rats were sacrificed on the same day. Skins of the rats were fixed in 20% formalin.

Fluorescein isothiocyanate (FITC) staining and flow cytometric (FCM) analysis

Isolated cells from either the thymus or MLN were adjusted to a concentration of 10^6 cells in 50 μl phosphate-buffered saline (PBS). One μg of anti rat T cytotoxic/suppressor cell monoclonal antibody (anti CD8 mAb)-FITC (Cedarlane Laboratories Limited, Canada, U.S.A.) was added to the cell suspension. The mixing of FITC-anti CD8 mAb and lymphocytes was incubated for 30 min at 4℃ and then added 1% paraformaldehyde to maintain at 4℃ overnight. After washing 2-3 times with PBS, the cell pellet was resuspended in 500 μl PBS-0.5% BSA. The cells were analyzed by an EPICS(R) Profile ll Analyzer (Coulter, U. S. A.). The percentage of fluorescent cells stained with FITC-anti CD8 mAb was calculated.

Immunohistological assay

A few MLN were fixed in 20% formalin. The sections were stained with immuno-alkaline phosphatase. Mouse mAb to rat thymocytes and T cells, that is, anti CD5 mAb (Serotec, England), was mounted on the sections for 50 min. Alkaline phosphatase conjugated anti rat IgG (Fc) (BSL, England) was used as an antiserum reacting to anti CD5 mAb. The sections were stained with naphthol AS-MX and Fast Red TR. They were counterstained with Mayer's hematoxylin. A trinitron color video monitor (Sony, Japan) was applied for $CD5^+$ cell counting. $CD5^+$ cell numbers were counted over an area of 0.0638 mm^2. The highest number among the counts was selected as the result.

Enzyme-linked immunosorbent assay (EIA)

Anti lysozyme antibody in all the rat plasma was measured by EIA. Falcon 3072 culture plate (Becton Dickinson, U.S.A.) was coated with 200 μg of chicken egg lysozyme per well. Fourty μl of plasma was added to each well. The plate was incubated for 2 h at 37℃ and overnight at 4℃.

Anti rat IgG (AM) conjugated with alkaline phosphatase (BSL, England) was added to the washed plate and incubated for 2 h at 37℃. Four-nitrophenyl phosphate Na salt was used as a substrate. The reactions were stopped with 1 N NaOH. Alkaline phosphatase activity was measured at 405 nm. The positive control was a well containing the substrate in alkaline phosphatase conjugated antiserum. The negative control was a well to which no plasma was added.

Table 1. $CD8^+$ and $CD5^+$ cell numbers counted in the lymphocytes of the rat thymus and MLN

Exp. (Sex)-Rat No.		$CD8^+$ T cell %		$CD5^+$ cell number*
		Thymus (M±SD)	MLN (M±SD)	MLN (M±SD)
A(M^s)	-1, 2, 4	90± 3	13± 1	19± 8
	-3	98	10	12
B(F^s)	-1, 5	88± 2	9± 1	14± 3
	-4	78	6	40
	-2, 3, 6	89± 5	14± 1	13±10
C($M^{s,c}$)	-1, 2, 3, 4	95± 0	12± 2	14± 5
	-5	93	7	9
D($F^{s,c}$)	-1, 3, 4, 5, 6	—	17± 1	18± 7
	-2	—	13	42
E(M^c)	-1, 2, 3, 5	98± 1	19± 2	4± 3
	-4	98	23	40
F($F^{s,l}$)	-1, 2, 3, 4, 5, 6	—	19± 3	18±11
G(F^l)	-1, 5, 6	—	16± 3	8± 5
	-2, 4	—	18± 2	35± 3
	-3	—	22	81
Cont(M)	-1, 2, 3, 4, 5, 6, 7	92± 5	16± 3	8± 5
Cont(F)	-1, 2, 3, 4, 5	95± 1	20± 2	20±10

*$CD5^+$ cell number in 0.0638 mm^2 area, ^{s}splenectomy, ^{c}chicken egg lysozyme injection, ^{l}levamisole injection.

Latex phagocytic test

Blood used in this test was taken by sodium citrate or heparin at sacrifice. Latex beads of 1.09 μm (Sigma Chemical Co., U.S.A.) were diluted to 0.02% with saline. White cell rich blood, which was obtained from Exp. D, F, G and control females, was mixed with 0.02% latex solution and then incubated for 60 min at 37℃. White cell rich blood separated from one control female was resuspended in plasma of her own, Exp. (Rat No.) D(3) rat and E(3) rat. Besides the latex phagocytic test, an another tube containing Exp. (Rat No.) D(3) rat plasma and the cells was incubated with rabbit complement (Behringwerk AG, Germany) for further 1 h at 37℃ to examine granulocytotoxic reaction. To examine PMN neutrophil functions, blood smears of each mixture were made and then were stained with May-Gruenwald Giemsa and peroxidase (Muto Pure Chemicals Co., Ltd., Japan). Phagocytic activities were examined in 100 PMN neutrophils. At the same time, the nuclear lobe number of PMN neutrophil was counted. More than seven lobes were judged to be hypersegmented lobes.

Table 2. Antibody activities to chicken egg lysozyme measured by EIA

Exp. (Sex)-Rat No.		Absorbances at 405 nm (M±SD)
A(M^s)	-1	0.23
	-2, 4	0.07 ± 0.03
	-3	0.34
B(F^s)	-1, 2, 3, 4, 5, 6	0.09 ± 0.06
C($M^{s,c}$)	-1	0.32
	-2, 3, 4, 5	0.24 ± 0.01
D($F^{s,c}$)	-1, 2, 5	0.32 ± 0.01
	-3	0.59
	-4, 6	0.24 ± 0.01
E(M^c)	-1, 2, 4, 5	0.21 ± 0.03
	-3	0.35
F($F^{s,l}$)	-1, 2, 3, 4, 5, 6	0.10 ± 0.05
G(F^l)	-1, 2, 3, 4, 5, 6	0.11 ± 0.04
Cont(M)	-1, 2, 3, 4, 5, 6	0.10 ± 0.04
Cont(F)	-1, 3, 4, 5, 6	0.13 ± 0.03
	-2	0.29
Negative control		0.01
Positive control		0.98

^{s}splenectomy, ^{c}chicken egg lysozyme injection, ^{l}levamisole injection.

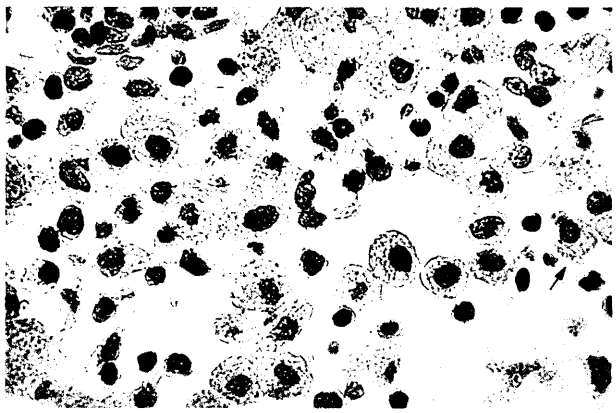

Fig. 1. The medullary sinuse of a G(3) rat MLN. Many $CD5^+$ cells have been stained faintly red with mouse anti CD5 mAb to rat thymocytes and T cells. Not only T cells but also non-T cells can be recognized as $CD5^+$ cells. Relatively small cells (shown by arrow) are activated B cells. The large cells are cells belonging to tissue mast cells. (Immuno-alkaline phosphatase, ×520)

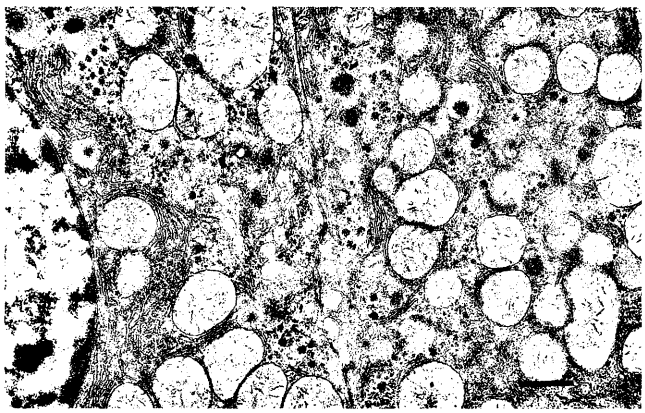

Fig. 3. The electron micrograph of a C(3) rat hepatocytes. Mitochondrial proliferation is remarkable. Many peroxisomes have an inclusion body. Rough endoplasmic reticulums are well developed at the perinulclear area. Dark line is 1 μm long. (Uranyl acetate-lead citrate double stain, ×7,200)

Electron microscopic analysis

The liver of Exp. (Rat No.) C(3) male was fixed with 2% glutaraldehyde for 2 h and then postfixed with 1% osmium tetroxide. Thin sections were examined by transmission electron microscope (JEOL 100CX).

RESULTS

A mild PCA reaction and blue spot were found in one of the four control males. No PCA reaction was

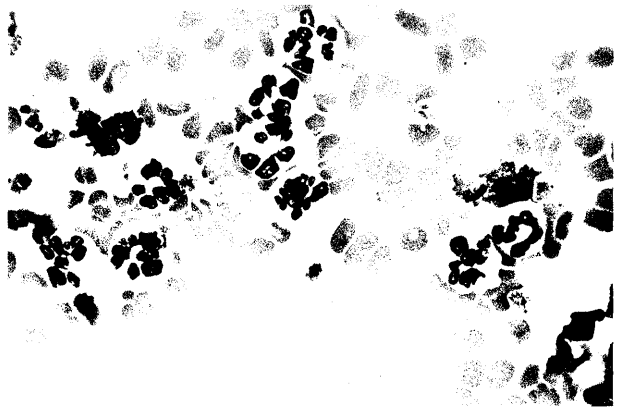

Fig. 2. The peripheral blood smear of a D(3) rat. Many hypersegmented PMN neutrophils with dense nuclear chromatin have not been stained with peroxidase. (May-Gruenwald Giemsa stain, ×520)

detected in splenectomized males of Exp. A, but a blue spot appeared at 20 min. Histopathological study of the control male skin showed a vacuolated edematous malpighian layer with mild lymphocyte infiltrations and keratinization of stratum granulosum with degradation of keratohyaline granules. Table 1 summarizes the $CD8^+$ and $CD5^+$ cell numbers counted in the thymus and MLN. One splenectomized female of Exp. B(4) had decreased $CD8^+$ T cell numbers in the thymus (78%). In five splenectomized rats of Exp. A(3), B(1, 4 and 5) and C(5), $CD8^+$ T cell numbers of MLN decreased to less than 10%. $CD5^+$ cells were detected in the juxtamedullary regions and medullary sinuses. More than 40 $CD5^+$ cells were counted in the 0.0638 mm^2 MLN areas of Exp. B(4), D(2), E(4) and G(3) rats. Exp. G(3) female had 81 $CD5^+$ cells in the MLN area of 0.0638 mm^2. A splenectomized B(4) female showed both $CD8^+$ T cell decrease and $CD5^+$ cell increase in the MLN. Fig. 1 demonstrates $CD5^+$ cells of MLN which were stained with immuno-alkaline phosphatase. Some $CD5^+$ cells were judged to be activated B cells. Tissue mast cells also reacted to anti CD5 mAb. Table 2 indicates plasma antibody activities to chicken egg lysozyme which were measured by EIA. Exp. C, D and E rats, which were immunized with chicken egg lysozyme, showed high antibody activities to the lysozyme. More than 0.30 absorbances were measured in Exp. A(3), C(1), D(1, 2, 3 and 5) and E(3) rats. The Exp. D(3) female had 0.59 absorbance. Moderately elevated absorbance of 0.29 was measured in one of the control females.

Table 3 summarizes the morphological and functional changes detected in PMN neutrophils. Females of Exp. D showed hypersegmented PMN neutrophils. Among them, the Exp. D(3) female had 81% hyperseg-

Table 3. Morphological and functional studies of PMN neutrophils

Exp. (Sex)-Rat No.		Nucleus with ≧7 lobes (M±SD) %	Phagocytosis of latex beads (M±SD) %	Peroxidase Stain
D($F^{s,c}$)	-1, 5, 6	43 ± 2	10 ± 7	(−)~(+)w*
	-2	56	5	(−)~(+)w
	-3	81	9	(−)
	-4	35	11	(±)~(+)w
F($F^{s,l}$)	-1, 2	22 ± 2	4 ± 1	(+)w
G(F^{l})	-1, 2, 3, 4, 6	32 ± 5	8 ± 3	(+)w
Cont(F)	-1, 2, 3, 4, 5	25 ±11	36 ±11	(+)w
	-6	15	42	(+)w
	-6 (in D-3 plasma)	24	3	(+)w
	-6 (in E-3 plasma)	26	5	(+)w

*weakly positive, ^{s}splenectomy, ^{c}chicken egg lysozyme injection, ^{l}levamisole injection.

mented PMN neutrophils (Fig. 2). Nuclear chromatin was dense in all the hypersegmented PMN neutrophils. Decreased phagocytosis of latex beads was found in the females of Exp. D, F and G. The disturbed phagocytosis was characterized not only by low percentages but also by low latex numbers in each PMN neutrophil. Only a few latex beads were phagocytized in the PMN neutrophils. When control PMN neutrophils were incubated in the plasma of Exp. D(3) and E(3) rats, PMN neutrophil numbers phagocytized latex beads decreased from 42% to 3-5%. A complement-dependent granulocytotoxic reaction of control PMN neutrophils was not induced in the plasma of the Exp. D(3) rat. Although the peroxidase reaction of PMN neutrophils was not strong in any of the rats, many hypersegmented PMN neutrophils of the Exp. D(3) female lost the peroxidase reaction completely. Tissue mast cells in the MLN of the Exp. D(3) female showed positive peroxidase reaction. Fig. 3 is the electron micrograph of Exp. C(3) male hepatocytes. A marked proliferation of mitochondria was characterized in some of the hepatocytes. Many peroxisomes containing an inclusion body were recognized among the mitochondria.

DISCUSSION

Viewed from the immunological changes of post-splenectomy, autoimmune reactions of PMN neutrophils were investigated in splenectomized Lewis rats immunized with either levamisole or lysozyme. In this study, $CD8^+$ T cell numbers of MLN were low in splenectomized rats, five of which had less than 10% $CD8^+$ T cells. PCA skin reaction of splenectomized rats to anti DNP-Ascaris was weaker than normal controls, in spite of the immediate blood circulation of the antigen injected into veins. CD8 antigen is known to work with the T cell receptor (TCR) to recognize the peptides derived from degraded cytoplasmic proteins, which were bound by class I molecules of major histocompatibility (MHC) antigens expressed on antigen-presenting cells.[6] Cellular immune responses mediated by $CD8^+$ T cells were shown to be disturbed in the splenectomized Lewis rats. CD5 is the antigen found on nearly all T lymphocytes and on a subpopulation of B cells. The proliferation of $CD5^+$ cells including B cells was found in juxtamedullary regions and medullary sinuses of the rats' MLN. As $CD5^+$ B cells are reported to be the primary source of autoantibodies to ssDNA and the Fc fragment of IgG,[7] autoimmunity to ssDNA and IgGFc was suspected to be triggered in the rats.

Decreased phagocytosis of PMN neutrophils was shown in the rats treated with levamisole or lysozyme. Normal PMN neutrophils also lost phagocytic functions when they were incubated in the plasma of Exp. D and E rats immunized with lysozyme. Autoantibody to the Fc fragment of IgG which was produced in $CD5^+$ B cells must have blocked the process of phagocytosis. The specific recognition of the particles must have been blocked at neutrophil Fc receptors by autoantibody to IgGFc. Levamisole and lysozyme triggered PMN neutrophils dysfunction based on autoimmunity to IgGFc. The rats injected with chicken egg lysozyme produced antibody to lysozyme. The highest titer of anti lysozyme antibody was measured in one of the splenectomized Exp. D females, whose blood smear showed many PMN neutrophils with hypersegmented hyperchromatic nucleus. A similar cross-reaction as in the P-ANCA reaction, namely the myeloperoxidase auto-cell reaction, was

considered to have been inducted in the PMN neutrophils of the rats. It was indicated by the decreased peroxidase stain of the hypersegmented PMN neutrophils. The impaired DNA replication might be associated with the H_2O_2 that was released when myeloperoxidase degranulation occurred in PMN neutropils.[8] Impaired DNA replication of PMN neutrophil nucleus was also suspected to be caused by autoantibodies to ssDNA or nuclear histone components.[9] Liver changes related to the PMN neutrophil autoimmunity were demonstrated in one of the Exp. C males immunized with lysozyme after splenectomy. Well developed rough endoplasmic reticulums were detected to the perinuclear area of the hepatocytes. Many peroxisomes were found in the hepatocytes, which was indication of the immature production of peroxidase, catalase and uricase. Mitochondrial proliferation in the hepatocytes seemed to be caused by surgical removal of the spleen, antibody production to lysozyme and directly by decreased respiratory polypeptides. The spleen, which was rich in lysozyme mRNA,[10] was concluded to play an important role in protein synthesis in liver.[2]

As levamisole usually activates macrophages, splenectomy effects on the rats treated with levamisole have not been evident. $CD5^+$ cells of MLN were increased in non-splenectomized females. Splenectomy effects on the rats treated with lysozyme were so evident as stated above that postsplenectomy macrophage dysfunction stated by JE McCarthy et al.[1] was suggested to be combined with autoimmunity to PMN neutrophils.

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