Bull Yamaguchi Med Sch 36(3-4) : 61—67, 1989

The Role of H-Y Antigen in Acute Graft-Versus-Host Responses

Tadako Nakatsuji and Tokuhiro Ishihara***

*Department of Transfusion and Clinical Immunology, Hamamatsu University School of Medicine, Hamamatsu 431-31, Japan
**The first department of Pathology, Yamaguchi University School of Medicine, Ube, Yamaguchi 755, Japan
(Received August 23, revised October 19, 1989)

Abstract Acute graft-versus-host reactions (GVHRs) to a male-specific (H-Y) antigen have been investigated using a weight assay, a bone marrow assay and a focal periportal proliferation test. Severe acute GVHRs have been induced by the injection of female DA rat cells into male (DAx Lewis) F_1 hybrid rats. Besides semiallogeneic donor RT1 antigens, host H-Y antigen was concerned strongly with leading to systemic acute GVHRs. Even though there were clinical variations, the acute GVHRs to H-Y antigen were occurred in F_1 males, especially by the immunization with DA female spleen cells. When F_1 hosts were splenectomized, only mild to moderate acute GVHRs were recognized by the injection of either female or male donor cells. The donor cells obtained from Lewis females that were preimmunized with male F_1 host cells demonstrated a paradoxical phenomenon in F_1 males. Among the 4 rats, one rat died of typical acute graft-versus-host disease (GVHD). But in the other 3, chronic graft rejection was suggested to add to the GVHRs. Hemolytic anemia and ballooning change of hepatocytes were developed at around 41-63 days after immunization. Acute GVHRs to H-Y antigen led to remarkable erythroid suppression on bone marrow and focal myeloid proliferation in liver.

Key Words : Acute GVHR, H-Y antigen, F_1 hybrid rat, Splenectomy, Erythropoiesis

Introduction

The male-specific (H-Y) antigen in mice, which is controlled by major histocompatibility complex (MHC) genes, triggers a complex of immunological responses[1]. Graft-versus-host (GVH) responses to H-Y antigen were observed in male F_1 hybrid rats after parental female lymphocytes were injected into the footpads with the activated macrophages[2]. The popliteal lymph node assay has been used to detect local T cell responses to the H-Y antigen. Anti-H-Y immunological responsiveness was examined in female mice by the immunization with syngeneic male spleen cells[3].

In this study, the role of H-Y antigen in systemic acute graft-versus-host reactions (GVHRs) has been examined. The interactions between MHC antigens and H-Y antigen were considered in respect of acute GVHRs. Although many assays have been reported for the measurement of GVH responses[4], the body weight assay[5], a bone marrow assay and a focal periportal proliferation test[6] were applied for this study. In the bone marrow assay, the non-erythroid to erythroid ratio (non-E/E) and mast cell %

were measured.

Materials and Methods

DA(RT1^a), Lewis(RT1^l) and (DAxLewis)F_1 hybrid rats of both sexes were used in this study. These rats which were originally supplied from John Curtin School of Medical Research, Canberra, Australia, were provided by Dr A. Yamashita. The host rats were 6.5-9.5 weeks old and weighed 90-150 g. They were raised in a clean environment on a standard diet. The detailed experimental protcols used in this study were shown in Table 1.

Host rat treatments (splenectomy and prenatal immunization) : Eight (DAxLewis)F_1 hybrid hosts were splenectomized at 6 week old and then immunized 2.5-3.0 weeks later (Exp. Nos. 1&3). The DA and Lewis cells were injected into pregnant Lewis females via tail vein. The immunized rats gave birth to total nine Lewis rats, 15-17 days after immunization (Exp. Nos. 8&9). The donor cells used for the prenatal immunization were DA male cells ($5.1x10^7$ spleen cells, $11.7x10^7$ lymph node cells, $6.8x10^7$ thymocytes & $4.7x10^7$ bone marrow cells per rat), and Lewis male cells ($38.4x10^7$ spleen cells per rat).

Donor cells and immunization : Male (DAxLewis)F_1 hybrid rats, male Lewis rats, splenectomized male (DAxLewis)F_1 hybrid rats and male Lewis rats immunized before birth were used as hosts. Basically, 5 kinds of donor cells were used for the immunization. (1) cell suspensions prepared from the spleens, cervical, axillary & mesenteric lymph nodes, thymuses and bone marrows of DA male rats (Exp. Nos. 1 & 2), (2) the same as in (1) but from DA female rats (Exp. Nos. 3, 4-A, B, 7 & 8), (3) cell suspensions prepared from DA female rats that had been subjected to splenectomy 2 weeks beforehand (Exp. No. 4-C), (4) cell suspensions prepared from a Lewis female rat which was presensitized with $10x10^7$-$18x10^7$ spleen cells obtained from F_1 males three times, at weekly interval, via footpads (Exp. No. 5), and (5) cell suspensions comprising the same as in (2), but which were mixed with $82.4x10^7$ Lewis female spleen cells (Exp. No. 6). Exp. No. 10-A and B were normal controls. The donor cells were suspended in either cold Hanks' solution or cold RPMI 1640 medium and then filtered through several layers of gauze. The suspensions were washed three times with the medium. The viability of the cells was more than 90%. It was judged from trypan blue test. The total cell counts in the cell suspensions ranged from $17.2x10^7$ to $97.4x10^7$ cells per rat. Each immunization was carried out via tail vein.

Assay : Mortality assay. The graft-versus-host disease (GVHD) rats have been observed until death. Weight assay. Rats were weighed every three days, for a total of 46-100 days, usually 80-85 days. Acute GVHRs, which began

Table 1 Experimental models used in this study.

Exp. No.	Recipiet strain	Donor strain	Cell suspension No.	Concentration ($x10^7$ cells/rat)	No. of rats	B.W. (g)	Days observed after injection	Figure referred
1	F_1♂[a]	DA♂	1	S(5.3)+L(11.0)+B(5.3)+T(5.3)	4	130	84	(Fig. 1)
2-A	F_1♂	DA♂	1	S(5.3)+L(11.0)+B(5.3)+T(5.3)	4	130	88	(Fig. 1)
2-B	F_1♂	DA♂	1	S(6.3)+L(17.2)+B(7.9)+T(8.3)	4	110	63	
3	F_1♂[a]	DA♀	2	S(6.9)+L(13.8)+B(5.1)+T(8.6)	4	150	81	(Fig. 2)
4-A	F_1♂	DA♀	2	S(6.9)+L(13.8)+B(5.1)+T(8.6)	3	150	81	(Fig. 2)
4-B	F_1♂	DA♀	2	S(6.1)+L(14.3)+B(4.2)+T(8.1)	5	130	74	(Fig. 3)
4-C	F_1♂	DA♀[a]	3	S(0)+L(8.9)+B(5.5)+T(2.8)	4	130	46	(Fig. 3)
5	F_1♂	Lewis♀[b]	4	S(13.5)+L(13.7)+B(5.0)+T(13.1)	4	120	100	
6	Lewis♂	DA♀ &Lewis♀	5	S(2.8)+L(6.8)+B(1.1)+T(4.3) S(82.4)	3	120	84	
7	Lewis♂	DA♀	2	S(9.2)+L(22.7)+B(6.7)+T(14.6)	4	140	80	
8	Lewis♂[c]	DA♀	2	S(9.2)+L(22.7)+B(6.7)+T(14.6)	5	90	80	
9	Lewis♀[c]	DA♀		Not inoculated	4	-	80	
10-A	F_1♀ (8.5 weeks)			No treatment	4	110	-	
10-B	F_1♂ (5.5 months)			No treatment	4	270	-	

[a]splenectomy, [b]preimmunization, [c]prenatal immunization.
S, spleen ; L, lymph node ; B, bone marrow ; T, thymus.
B.W., initial body weight with the deviations of less than 15 g.

to occur around 8 days after cell injection, were diagnosed on the basis of clinical manifestations, e.g. erythema of the ears, tail, face and legs, followed by petechiae, erosion & scale formation of the skin, allergic conjunctivitis, stomatitis, diarrhea and weight loss. Bone marrow assay. Bone marrow cells which were collected at the time of sacrifice were washed in Hanks' solution or RPMI 1640 medium two or three times. To make smears, $1x10^5$ bone marrow cells suspended in 100 μl of the medium were centrifuged at 800 r.p.m. for 5 min in a Shandon cytospine 2. Alternatively, concentrated bone marrow cells were smeared directily onto slide glasses. The bone marrow smears were stained with May-Gruenwald-Giemsa. To calculate non-erythroid to erythroid ratio (non-E/E) and mast cell %, at least one thousand cells were counted. Non-erythroid cells indicated all the nucleated cells except for erythroid cells in bone marrow. The ratios of mast cells to total nucleated cells (non-E+E) were calculated to indicate mast cell %. Focal periportal proliferation test. Liver specimens were either frozen in O.C.T. medium (Ames) for cryostat sectioning or fixed in neutral formalin for paraffin embedding. Liver sections were subjected to routine hematoxylin and eosin (H & E) staining. Beta-glucuronidase staining, the Berlin blue reaction and periodic acid-Schiff (PAS) reaction were also in use when necessary.

The spleens of Exp. No. 5 were fixed in formalin for routine H & E staining.

Results

Figs. 1-3 show the results of the weight assays during 47 days after inoculation. The initial body weights of the rats were not exactly the same, but for simplicity, the averages of the initial weights were used in Figs. 1, 2 and 3. In Fig. 1, the weight changes of Exp. No. 1 rats (1,2,3,4) and Exp. No. 2-A rats (5,6,7,8) are presented. The splenectomized rats in Exp. No. 1(1,2,3,4) indicated slight weight losses at 11-20 days after injection. The non-splenectomized rats in Exp. No. 2-A(5,6,7,8) showed normal weight gains. The rats in Exp. No. 2-B also showed normal weight gains, except for one which had an episode of chronic GVHD (no Fig.). Chronic GVHD signs were recognized at 43 days after cell immunization. Fig. 2 points out the results of the weight assays in Exp.

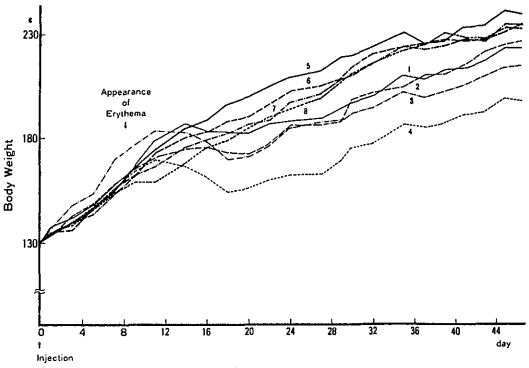

Fig. 1 Results of the weight assays in Exp. No. 1(1,2,3,4) and Exp. No. 2-A(5,6,7,8). This figure shows the differences in body weight changes between splenectomized F_1 hosts (Exp. No. 1) and non-splenectomized F_1 hosts (Exp. No. 2-A). In both experiments, the same DA male cells were used as donor cells. The day when donor cells were injected into the hosts was taken as day 0.

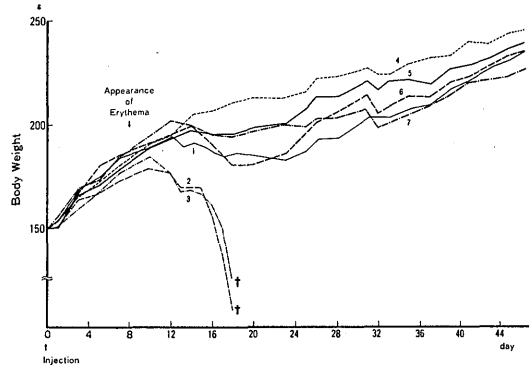

Fig. 2 Results of the weight assays in Exp. No.3 (4,5,6,7) and Exp. No. 4-A(1,2,3). This figure shows the apparent differences in acute GVH responses to the H-Y antigen between splenectomized F_1 hosts (Exp. No. 3) and non-splenectomized F_1 hosts (Exp. No. 4-A). In both experiments, the same DA female cells were used as donor cells. Two of the Exp. No. 4-A rats died of the acute GVHD.

No. 3 rats (4,5,6,7) and Exp. No. 4-A rats (1, 2,3). Among the three non-splenectomized rats of Exp. No. 4-A, two began to show weight losses at 10 days after immunization and died of the acute GVHD at 18 days after cell injection. However, all the splenectomized rats in Exp. No. 3 showed slight weight losses between 13 and 20 days after immunization. This pattern is similar to that of Exp. No. 1. Fig. 3 demonstrates the results of weight assays in Exp. No. 4-B(1,2,3,4,5) and Exp. No. 4-C(6,7,8,9). These two experimental systems were basically the same as that in Exp. No. 4-A. In the five rats of Exp. No. 4 -B, one died of acute GVHD and two rats showed severe clinical signs of acute GVHD, which were followed by gradual spontaneous recovery. The other two showed moderate acute GVHD signs. All the rats of Exp. No. 4 -C had mild suppression of weight gain and mild GVH symptoms. Among the 4 rats of Exp. No. 5, one rat indicated the typical pattern of acute GVHD and the others had the fluctuations in body weight at around 41 and 63 days after immunization. Somewhat depressed weight gains were recognized in

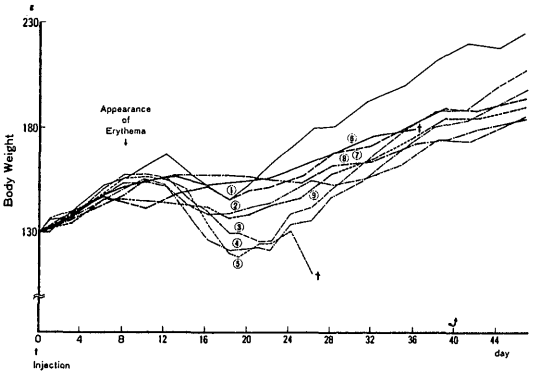

Fig. 3 Results of the weight assays in Exp. No. 4-B(1,2,3,4,5) and Exp. No. 4-C (6,7,8,9). This figure shows the different body weight changes based on the severities of acute GVHRs. Both experimental systems were basically the same as that in Exp. No. 4-A. One of the Exp. No. 4-B rats died of the acute GVHD and one of the Exp. No. 4-C rats died from unknown causes.

Table 2 The results of the mortality assay and bone marrow assay.

Exp.No.	Mortality rate (Death/Total)	No. of rat assayed	Bone marrow findings Non-E/E ($\bar{M}\pm SD$)	Mast cell % ($\bar{M}\pm SD$)
1	0/4	4	3.8 ±0.8	1.6 ±0.2
2-A	0/4	4	2.4 ±0.2	1.1 ±1.1
2-B	0/4	4	2.5 ±0.5	1.3 ±0.7
3	0/4	4	3.7 ±0.3	1.5 ±0.8
4-A	2/3[a]	1	4.0	3.3
		1	44.8[b]	0[b]
4-B	1/5	4	3.1 ±0.5	3.3 ±0.8
		1	3.9[b]	3.7[b]
4-C	1/4[a]	3	2.0 ±0.3	2.8 ±0.3
5	1/4	3	2.1 ±0.5	1.2 ±0.7
		1	32.7[b]	0.2[b]
6	0/3	3	1.8 ±0.6	0.3 ±0.1
7	0/4	4	2.3 ±0.3	0.3 ±0.1
8	0/5	5	2.9 ±0.2	0.4 ±0.1
9	0/4	4	3.4 ±0.4	0.3 ±0.1
10-A	0/4	4	3.8 ±0.2	0.2 ±0.1
10-B	0/4	4	3.4 ±0.1	0.3 ±0.1

[a]In one rat of Exp. No. 4-A and one rat of Exp. No. 4-C, which died of acute GVHD and an unknown cause, respectively, bone marrow assay was not carried out.
[b]Bone marrow findings of the rat died of acute GVHD.

the rats of Exp. Nos. 6 and 7, while the rats of Exp. No. 8 had normal weight gains. Exp. Nos. 6,7,&8 rats did not induce acute GVHD signs clearly.

The results of the bone marrow assay are presented in Table 2. The two rats with acute GVHD showed remarkable increase in non-E/E (32.7-44.8). Myeloid cell populations were predominant. Erythroid cell populations were severely suppressed in the bone marrows. Fig. 4 demonstrates a picture of undifferentiated mesenchymal stem cells (hemohistioblastosis) surrounded by many myeloid cells, like "erythroblastic islands". It was recognized in the Exp. No. 5 rat with acute GVHD. The two rats with acutely lethal GVHRs to H-Y antigen had the disappearance of bone marrow mast cells. The Exp. No. 4-B (5) rat which died of the acute GVHD but showed recovery signs for several days (Fig. 3), indicated a slightly increased non-E/E (3.9) and an elevated mast cell % (3.7%). In the bone marrow of rats showing not lethal but acute GVH responses to the H-Y antigen (Exp. Nos. 4-A, B & C), mast cell % was increased to $2.8\pm0.3\%-3.3\pm0.8\%$ (Table 2). Mild to moderate GVH responses to semiallogeneic RT1 antigens led to a decreased non-E/E ($2.0\pm0.3-2.5\pm0.5$) with a slight increase in mast cell % (Exp. Nos. 2-A &B, 4-C and 5). Their bone marrow contained, at least relatively, increased numbers of "erythroblastic islands". The splenectomized rats had $3.7\pm0.3-3.8\pm0.8$ of non-E/E and $1.5\pm0.8\%-1.6\pm0.2\%$ mast cells. When DA female cells were injected into Lewis males, there were a decreased non-E/E ($1.8\pm0.6-2.9\pm0.2$) and a normal mast cell % ($0.3\pm0.1-0.4\pm0.1$), which resulted from Exp. Nos. 6, 7 and 8. In the rats immunized with both DA and Lewis male cells at the fetal stage (Exp. No. 8), non-E/E was 2.9 ± 0.2, compared with the 2.3 ± 0.3 of the non-immunized rats at the fetal stage (Exp. No. 7) and the 3.4 ± 0.4 of the control rats immunized at only the fetal stage (Exp. No. 9).

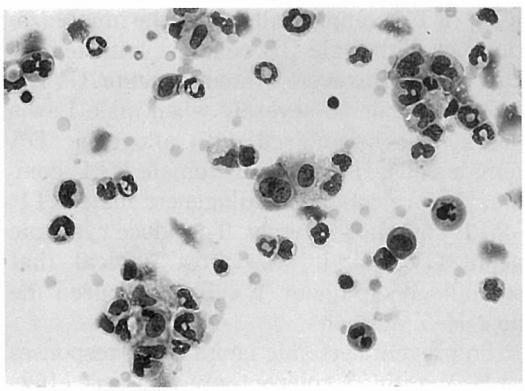

Fig. 4 Bone marrow smears prepared from the Exp. No. 5 rat. This rat died of the acute GVHD. The smear was made using a Shandon cytospine 2. Myeloid hyperplasia and depression of erythropoiesis are apparent. Hemohistioblasts are surrounded not by erythroblastic but by many myeloblasts. The "myeloblastic islands" are apparent in this smear. (May-Gruenwald-Giemsa stain ; x320).

The histopathological findings in immunized rat livers are summarized in Table 3. Strong lymphocyte migration and intraluminal projection of bile duct cells in the portal areas were specific findings in the livers of the rats with acutely lethal GVHRs to H-Y antigen. As shown in Fig. 5, focal periportal proliferation of myeloid cells was also observed in the rat liver with acute GVHD. On β-gluculonidase staining, increased numbers of phagocytic histiocytes and the mobilization of Kupffer cells were found in the liver. Mononuclear cells that had proliferated into or around the portal veins had made small foci in all the rats immunized with semiallogeneic cells. The livers of all the splenectomized rats and of the rat with chronic GVHD (Exp. No. 2-B) showed mild deposition of hemosiderin, which was detected by means of the Berlin blue reaction. The ballooning changes of hepatic cells were found in the rats immunized with presensitized female Lewis cells (Exp. No. 5). The ballooning hepatocytes showed a negative reaction on PAS staining. The clinical signs, changes in body weight, bone marrow findings and liver histopathological findings were all similar in splenectomized hosts, regardless of whether the donor cells were prepared from male or female DA rats. Increased deposition of hemosiderin was found in the spleens of Exp. No. 5 rats, except for the acute GVHD rat.

Table 3 Histopathological findings in the rat livers.

Histopathological findings (H & E stain)	Exp. No. 1 (n=4)	2-A,B (n=8)	3 (n=4)	4-A[a],B[a] (n=5)	4-C (n=3)	5[a] (n=3)	6 (n=3)	7 (n=4)	8 (n=5)
Focal periportal proliferation	+	±~−	+	+	+	−	−	±~−	±
Piecemeal necrosis	+	−	+	+	−	+	−	−	−
Hyperplasia of bile ducts	+	−	+	±~−	−	−	−	−	−
Lymphocyte migration in portal areas	−	−	−	+	+	−	−	−	−
Deposition of hemosiderin	+	−[b]	+	−	−	−	−	−	−
Hepatocellular ballooning	−	−	−	−	−	+~+++	−	−	−
Findings in acute GVHD	Lymphocyte migration and intraluminal obstruction of bile ducts in portal areas (+++) with piecemeal necrosis (+++), focal periportal infiltration with ectopic myelopoiesis (++~+++), mobilization of Kupffer cells (++).								

−, not pathological ; ±, slightly affected ; +, pathological changes. n, examined rat number.
[a]The rat liver findings in the acutely lethal GVHD were omitted. Listed below separately.
[b]A rat with a hemolytic episode due to the chronic GVHD showed deposition of hemosiderin.

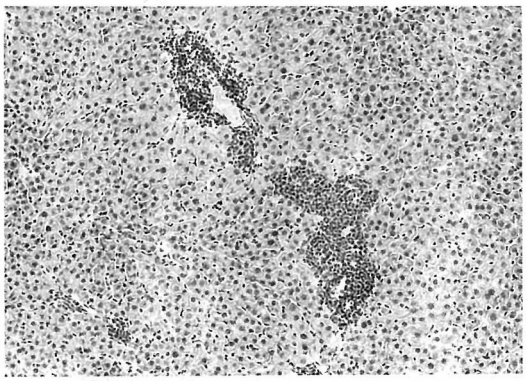

Fig. 5 Specimen prepared from the frozen liver of the Exp. No. 4-B rat. This rat died of the acute GVHD. Ectopic myelopoiesis is found in the portal areas or around the branches of portal veins. On the upper side of the figure, lymphocyte migration in the portal area is also seen. Intraluminal obstruction of bile ducts is apparent. Kupffer cells were mobilized in this liver. (H & E stain ; x120).

Discussion

The DA rat has the A (a) Pa (a) F (a) B (a) D (a) E (-) G (-) C (a) haplotype of the RT1 allele, while the Lewis rat has the A (l) Pa (-) F (a) B (l) D (l) E (-) G (a) C (l) haplotype[7]. Between these two strains, both class I and class II antigens are not identical. On the other hand, the gene regulation of the H-Y antigen has been investigated in mice. One gene located in the H-2K or H-2D region, Ir genes in the IA and IC regions and the gene in the Tarα-locus are considered to be necessary for the occurrence of anti-H-Y cytotoxity[1]. On this study, acutely lethal GVHRs were detected in male F_1 hosts with $RT1^a$-$RT1^l$ complex alleles by the immunization not with male DA cells but with female DA cells. However, systemic acute GVHD was not occurred severely when male Lewis hosts were immunized with allogeneic DA female cells. Homozygous female $RT1^a$ complex had rejected semiallogeneic male $RT1^a$-$RT1^l$ complex strongly. To induce systemic acute GVHRs, it was most critical that semiallogeneic donor T cells recognized the host H-Y antigen.

To prevent systemic acute GVH responses to H-Y antigen, splenectomy was very effective. Splenectomezed rats did not show acutely lethal GVHRs to H-Y antigen (Exp. No. 3, Fig. 2). On the other hand, acute GVHRs to H-Y antigen were suppressed in F_1 males, when DA spleen cells were excluded from DA donor cell mixtures (Exp. No. 4-C). It had been reported that splenic NK cell activity was increased at 8-16 days after the induction of acute GVHRs[8]. Removing the spleen from the host before immunization will result

in the suppression of host NK cell activity. For the explanation of the results obtained from this study, it was suggested that splenic NK cell removal in hosts might work to depress the acute GVHRs to H-Y antigen, as well as the donor cells in which spleen cells were excluded.

Recently, the production of high titer H-Y antibodies was demonstrated with the immunization method of intrasplenic implantation of syngeneic male skin[9]. The Lewis female cells preimmunized with male F_1 spleen cells might produce the anti-H-Y antibody and the $RT1^a$ antibody in F_1 males. Actually, the hosts of Exp. No. 5 showed body weight fluctuations at 41 to 63 days after inoculation, together with hemosiderin deposition in the spleen. The ballooning changes of hepatic cells in Exp. No. 5 rats seemed to have relations whth anti-H-Y and anti-$RT1^a$ immunological reactions, rather than to viral infection. Anti-H-Y immunological responsiveness was observed in CBA females that had been previously immunized with the syngeneic H-Y antigen when the first immunization was by i.p. injection[3]. The occurrence of anti-H-Y immunological responsiveness was demonstrated by the finding that the immunized female mice did not reject syngeneic male skin grafts. In this study, only one of the 4 rats in Exp. No. 5 developed acute GVHD. Besides host-versus-graft (HVG) responses to preimmunized donor cells, anti-H-Y immunological responsiveness had rather reacted to prevent the other 3 rats from developing the acutely lethal GVHRs to H-Y antigen.

For the prevention of GVHRs, prenatal immunization was also effective. The male Lewis fetuses ($RT1^l$) immunized with male DA ($RT1^a$) cells and male Lewis spleen cells were tolerant to the $RT1^a$ antigens. When the tolerant Lewis males were immunized once more with female DA cells after birth, the GVHRs to H-Y antigen were almost negligible. The incomplete tolerance was identified by these three GVHD assays (Exp. No. 8 in Table 2 & 3).

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.
The auther is grateful to Dr Akira Yamashita for scientific advice.

References

1) Epstein, R., Sham, G., Womack, J., Yagüe, J., Palmer, E. and Cohn, M. : The cytotoxic T cell response to the male-specific histocompatibility antigen (H-Y) is controlled by two dominant immune response genes, one in the MHC, the other in the Tarα-locus. *J. Exp. Med.,* **163** : 759-773, 1986.
2) Yamashita, A., Hattori, Y., Mori, F., Tamokoshi, K. and Fukumoto, T. : Generation of anti-H-Y reactive T cells in vivo by the addition of activated macrophages. *Transplantation,* **39** : 629-633, 1985.
3) Juretić, A. : Influence of the combination of intraperitoneal and footpad immunizaztion on anti-H-Y immunological reactivity. *Immunology,* **59** : 401-404, 1986.
4) Klein, J. : *Natural History of the Major Histocompatibility Complex.* 1st ed., A Wiley-Interscience Publication, New York. 1986, pp. 291-422.
5) Russell, P. S. : The weight-gain assay for runt disease in mice. *Ann. N.Y. Acad. Sci.,* **87** : 445-451, 1960.
6) Bain, G. O., Suen, W. Y., Remington, D. W. and Pinno, E. : Liver infiltration in the graft-versus-host reaction. *Transplantation,* **8** : 83-86, 1969.
7) Gill, T. J. III, Kunz, H. W., Misra, D. N. and Hassett, A. L. C. : The major histocompatibility complex of the rat. *Transplantation,* **43** : 773-785, 1987.
8) Ghayur, T., Seemayer, T. A. and Lapp, W. S. : Kinetics of natural killer cell cytotoxicity during the garft-versus-host reaction. *Transplantation,* **44** : 254-260, 1987.
9) Bradley, M. P. and Heslop, B. F. : Elicitation of a rapid and transient antibody response to H-Y antigen by intrasplenic immunization. *Transplantation,* **39** : 634-638, 1985.