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Regulation of experimental autoimmune orchitis by the presence or absence of testicular antigens during immunological development in SCID mice reconstituted with fetal liver cells

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SUMMARY

Severe combined immunodeficient (SCID) mice were immunologically reconstituted by the transfer of fetal liver cells (FLC) of BALB/c mice (SCID-FLC mice). In peripheral blood (PB) of SCID-FLC mice, B and T cells started to appear 2 and 5 weeks, respectively, after the transfer of FLC, and had attained normal levels by 7 weeks. Orchidectomy and transplantation of testis under the kidney capsule were conducted at various stages of immunological maturation, and the induction of experimental autoimmune orchitis (EAO) was performed after immunological maturation in SCID-FLC mice. The experimental system was used to establish that the presence of testicular antigens in the early stage of immunological development influences the induction of EAO; grade of EAO was reduced in the presence of the antigens, and enhanced in their absence. In other words, the existence of self tissue antigens in the early stage of immunological development was essential for proper establishment of tolerance to the self tissue. These findings suggested that the SCID-FLC mouse is a suitable model with which to analyse the interaction between self antigens and cells of the developing immune system, which is otherwise observed only in the fetal or perinatal stage in experimental animals.

INTRODUCTION

Although autoimmune diseases are mostly multifactorial, involving genetics, the environment and life style, one of their major causes is a disruption of unresponsiveness or immunological tolerance to self antigens. Burnet proposed that self-tolerance is acquired by elimination of self reactive clones after contact between the immune system and self antigens during fetal development.[1] Lederburg indicated that responsiveness is not determined by the developmental stage of the individual, but rather the state of maturity of the lymphocyte at the time it encounters antigen.[2] Thereafter, it was found that self-reactive clones were not necessarily deleted but often became anergic after contact with self antigens.[3] At any event, the presence of self antigens is vital to the establishment of self-tolerance. Thus, one can assume that removal of an organ from a fetus leads to a disruption of the self-tolerance for that organ after birth.

In 1962, Triplett[4] reported that ablation of pituitary gland in a larval frog inhibited the establishment of self-tolerance to the same pituitary gland, resulting in its rejection when grafted into the adult frog. The experiment clearly showed that deletion or anergy of self-reactive clones to pituitary gland did not occur when tissue antigens were absent during the larval stage.

Later in 1988, Eishi and McCullagh[5] tested Triplett's finding in the rat. They destroyed the fetal thyroid gland by injecting radio-iodine at 17 days gestation (the age at which the thyroid first becomes capable of concentrating iodine), with the result that autoimmune thyroiditis was induced when syngeneic thyroid tissue was transplanted into matured recipients. The development of T cells autoreactive to self thyrocytes was recently reported in lambs thyroidectomized during the fetal stage.[6]

In all of these cases, the experimental systems were relatively complicated and the removal of an organ or tissue antigens is by no means any easy procedure. Further elucidation of the mechanism of self-tolerance will require precise analysis of the cells of the immune system during larval or fetal stage. From a technical viewpoint, however, it is extremely difficult to manipulate the immune system during fetal development.

In this regard, we postulated that acquisition of self-

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Abbreviations: BP, *Bordetella pertussis*; CFA, complete Freund's adjuvant; EAO, experimental autoimmune orchitis; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; FLC, fetal liver cells; GD, gestation days; HE, haematoxylin and eosin; IFA, incomplete Freund's adjuvant; i.v., intravenous, PB, peripheral blood; PE, phycoerythin; SCID, severe combined immunodeficiency; SCID-FLC, SCID mice reconstituted by the transfer of FLC; SEM, standard error of the mean; SV, simian virus.

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tolerance is dependent upon the degree of immunological development, and not on the stage of somatic maturation. Severe combined immunodeficient (SCID) mice lack both T and B cells, but the immune system can develop within 7 weeks of the transfer of fetal liver cells (FLC) from normal BALB/c mice. Thus, we assumed that the process of acquisition of self-tolerance can be observed during gradual development of the immune system in SCID mice transferred with FLC.

In the present study, we first examined the development of the immune system in SCID mice after the transfer of FLC (SCID-FLC). Then, selecting testis as a target organ, we elucidated whether the presence of testicular tissue influenced the degree of experimental autoimmune orchitis (EAO) in SCID-FLC mice at different stages of immunological maturation. The results indicate that presence of testicular tissue in the early stage of immunological maturation significantly influences the grade of EAO and that SCID-FLC mice are a suitable experimental model with which to analyse the interaction of the developing immune system and self-antigens, which generally occurs at the fetal or perinatal stage. Finally, we briefly discuss the mechanism of acquiring self-tolerance during fetal development.

MATERIALS AND METHODS

Mice

SCID C.B-17/Icr-scid Jcl (SCID) and BALB/cAJcl (BALB/c) mice were used. All mice were supplied by Clea Japan, Tokyo, Japan, and maintained at the Tokyo Medical and Dental University.

Transfer of FLC to SCID mice

Livers removed from fetuses at gestation day (GD) 14 were used for preparation of free cell suspension of fetal liver cell (FLC). SCID mice were anaesthetized by intraperitoneal injection of 0·5 ml of 5% Nembutal (Dabbott co., Osaka, Japan) in saline, then injected intravenously into the tail with 1×10^7 FLC suspension.

Immunofluorescence staining of lymphocytes and fluorescence-activated cell sorter (FACS) analysis

Peripheral blood (PB) (100 μl) was collected from SCID-FLC mice every week after the transfer of FLC. PB was stained with phycoerythrin (PE)-anti-CD4 and either fluorescein isothiocyanate (FITC)-anti-CD8 or FITC-anti-immunoglobulin. These monoclonal antibodies were purchased from Becton Dickinson, CA. Flow cytometry analysis was performed with a FACScan (Becton Dickinson, CA).

Quantification of serum immunoglobulins

Enzyme-linked immunosorbent assay (ELISA) was employed for assessment of serum immunoglobulins, by using affinity-isolated rabbit anti-mouse immunoglobulin (Dakkopatts, SA, Denmark), biotin-conjugated rabbit anti-mouse immunoglobulin (Dakkopatts), and horseradish peroxidase-conjugated streptavidin (SAPx) (Dakkopatts). The enzymatic reaction was stopped by addition of 25 μl of 2N HCl, and absorbance of each well was measured at 490 nm with a microplate-reader (MTP-22 microphotometer, Corona Electric, Japan).

Orchidectomy

The lower abdominal hair of mice anaesthetized with Nembutal was cut and, a ventral midline incision about 1 cm in length made above the position of the preputial gland. Both testes were exposed by grasping the associated fat with forceps. The vas deferens and vessels were ligated and cut, and the testes then removed.

Testicular implantation

Testes were removed from 1-week-old male BALB/c mice, and the fat, epididymis and vas deferens then separated from the testes gently with forceps in saline. Freshly prepared testis of 1-week-old BALB/c mice was implanted under the renal capsule. The hair of the right side of the abdomen of anaesthetized mice was removed with a razor. An antero-posterior incision about 1 cm in length was made in the loin above the position of the right kidney, and the kidney exposed. The testicular tissue was introduced through the capsular incision and displaced to reduce the risk of subsequent loss through the incision. The kidney was replaced in its original position, and the abdominal wall closed with a single suture through both the skin and peritoneum.

Induction of EAO

The testes were removed from mice, teased with scissors in saline, and pumped several times with an 18G needle syringe. The suspension was homogenized with a ultrasonic crusher. The homogenate was adjusted with saline so that one testis (0·6–0·7 g) was contained in 0·6 ml of volume, then emulsified with an equal volume of complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA). Mice anaesthetized with Nembutal were subcutaneously injected with testicular emulsion into the four footpads then given an intravenous (i.v.) injection of 1×10^9 *Bordetella pertussis* (BP) in 0·3 ml of saline.

Histological grading of EAO

The original testis or the testis transplanted under the renal capsule was removed, and fixed in Bouin's solution for 3 hr. After embedding in paraffin, semiserial sections were cut at 4 μm and stained with Mayer's haematoxylin and eosin (HE). EAO of transplanted (Fig. 1) or original testis was graded from 0 to 5 on histological sections according to the degree of inflammation as follows: grade 0, no inflammation in testicular tissue (Fig. 1a,b), grade 1, occasional foci of lymphocytic infiltration (Fig. 1c), grade 2, multiple foci of lymphocyte infiltration accompanied by some follicular destruction, grade 3, coalescence of foci of lymphocytic infiltration (Fig. 1d), grade 4, coalescence of multiple foci of lymphocytic infiltration leading to development of a continuous area of infiltrate, grade 5, lymphocytic infiltration and/or necrosis of the entire testicular tissue (Fig. 1e,f).

RESULTS

Development of the immune system in SCID mice after the transfer of FLC (Fig. 2)

To follow the development of the immune system in SCID mice, animals between 5 and 9 weeks of age were reconstituted with 1×10^7 FLC from normal fetal BALB/c mice of GD 14. Every week after the transfer of FLC, 0·1 ml of PB was

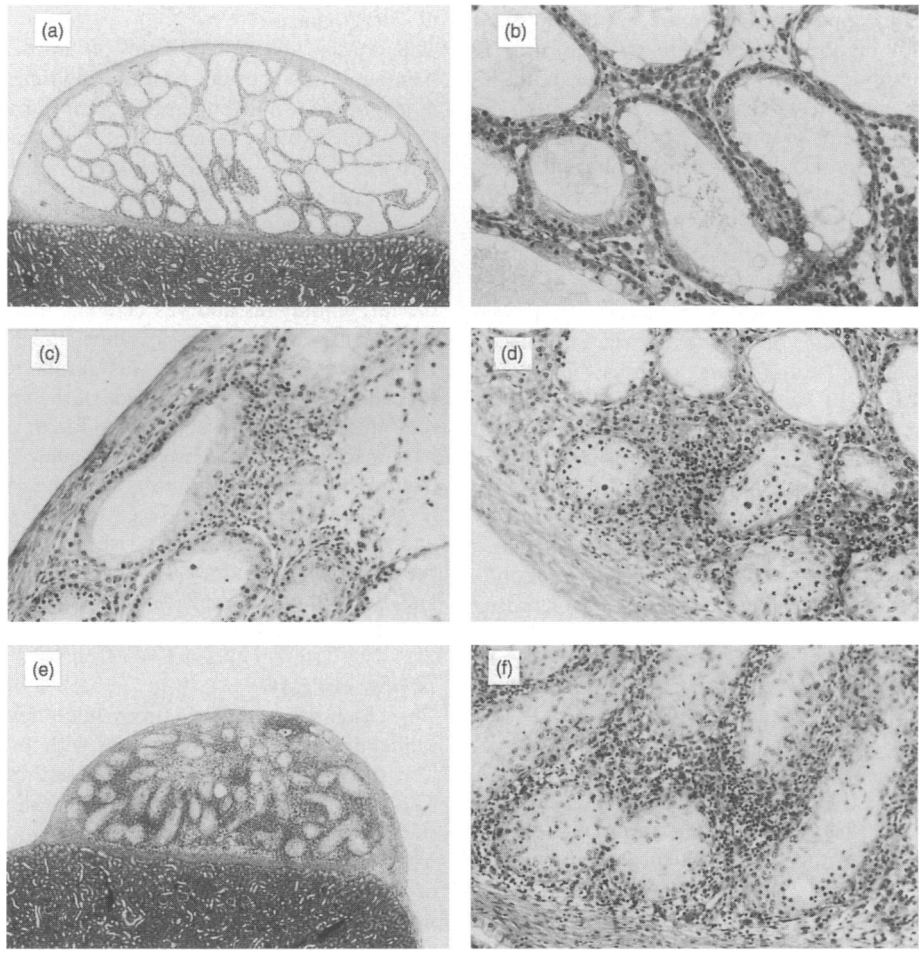

Figure 1. Histological grading of EAO in the testis grafted under renal capsule of mice by HE staining. (a) (×40) and (b) (×200): grade 0, no inflammation in testicular tissue. Atrophic tubules lined by only Sertoli cells. Degeneration and desquamation of spermatogonia and Sertoli cells without inflammatory reaction. (c) (×200): grade 1, occasional foci of lymphocytic infiltration. (d) (×200): grade 3, coalescence of foci of lymphocytic infiltration. (e) (×40) and (f) (×200): grade 5, lymphocytic infiltration of entire testicular tissue.

collected from the reconstituted mice to monitor the generation of peripheral populations of T cells ($CD4^+$ and $CD8^+$) and B cells (surface $immunoglobulin^+$), as well as serum concentration of immunoglobulins.

In the PB, B cells (surface $immunoglobulin^+$) started to appear 2 weeks after the transfer of FLC, increased gradually in number and reached the level of normal BALB/c mice in 5 weeks. T-cell populations ($CD4^+$ and $CD8^+$ cells) started to appear 5 weeks after the transfer of FLC, increased gradually in number and reached the level of normal BALB/c mice in 7 weeks. Serum immunoglobulin became detectable 2 weeks after transfer, and reached the level of normal BALB/c mice in 7 weeks (Fig. 2). Hereafter, SCID mice reconstituted with FLC are referred to as SCID-FLC mice.

Induction of EAO in BALB/c and SCID-FLC mice (Table 1)

EAO was induced by immunization of testicular antigens with CFA followed by i.v. injection of BP in non-treated BALB/c mice and non-treated SCID mice, as well as SCID-FLC mice immunologically reconstituted 12 weeks after the transfer of FLC. There was no significant difference in the grade of EAO in original testes between SCID-FLC mice ($3{\cdot}4 \pm 0{\cdot}7$) and BALB/c mice ($2{\cdot}6 \pm 0{\cdot}4$). Untreated SCID mice ($n=5$) showed total absence of any inflammatory lesions in original testes after induction of EAO.

EAO was also induced in the testis transplanted under the renal capsule of BALB/c and SCID-FLC mice, both of which had been previously orchidectomized. The grade of EAO in the transplanted testes was less severe than that of the original testes when immunized in the same manner; i.e. orchidectomized BALB/c mice, grade $1{\cdot}3 \pm 0{\cdot}1$ and orchidectomized SCID-FLC mice, grade $1{\cdot}3 \pm 0{\cdot}1$ (Table 1). Immunization of testicular antigens with CFA followed by i.v. injection of BP was essential for induction of EAO both in original and transplanted testis (Table 1).

Absence of testicular tissue during immunological development promotes induction of EAO in the testis transplanted in SCID-FLC mice

Findings so far presented indicated that EAO could be induced in both original and transplanted testes, but that inflammatory lesions are less severe in the transplanted testes. So we tested

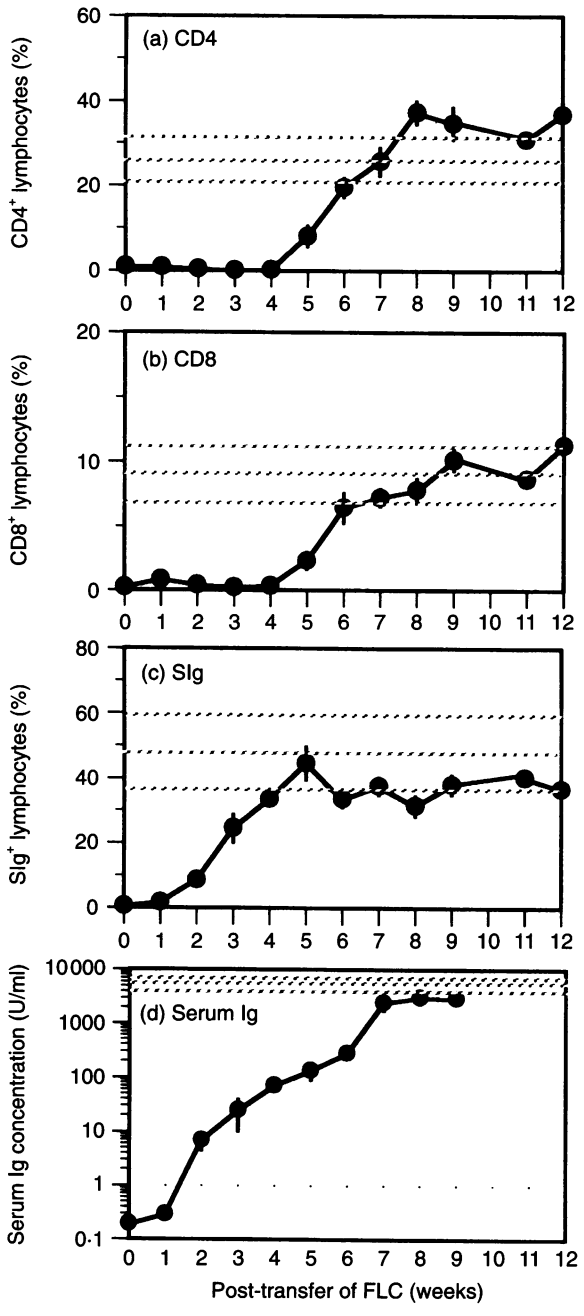

Figure 2. Development of peripheral lymphocyte populations and serum immunoglobulin level in SCID-FLC mice. The number of weeks after i.v. injection of the FLC is shown on the abscissa. The ordinate shows percentages of lymphocytes positive for each serum-marker, or serum immunoglobulin concentration. Each value (●) is the mean of levels in 10–12 SCID-FLC mice. Vertical lines, mean ± standard error of the mean (SEM). Horizontal dotted lines indicate mean ± SEM of level in 11 normal BALB/c mice of 10–12 weeks-of-age.

whether orchidectomy performed at various stage of immunological development influenced the degree of EAO of transplanted testis in SCID-FLC mice. In other words, absence of tissue antigens during the early stage of immunological development might interfere with the establishment of self-tolerance in the later stage, and therefore influence the degree of EAO.

The first experiment (Fig. 3) was conducted as follows. SCID mice were orchidectomized 2–4 weeks before or 0, 1, 2, 5, 8 and 12 weeks after the transfer of FLC. From weeks 9–13 after the transfer, transplantation of testicular tissue from BALB/c mice was carried out in these orchidectomized SCID-FLC mice. As observed in Fig. 2, SCID-FLC mice at this stage were immunologically mature. In SCID-FLC mice orchidectomized 12 weeks after the transfer, the testis was transplanted 15–16 weeks after the transfer. The transplanted testis was maintained for a period of more than 3 weeks before the induction of EAO. As controls, previously orchidectomized adult BALB/c and SCID mice received testicular transplantation and were immunized 2 weeks later.

Relative to the transplanted testis in SCID-FLC mice orchidectomized 12 weeks after the transfer of FLC, the grade of inflammation was significantly enhanced in the transplanted testis of the SCID-FLC mice orchidectomized 2–4 weeks before or 0–2 weeks after the transfer of FLC (Fig. 4). In contrast, the grade of inflammation in the transplanted testis was not significantly enhanced in the SCID-FLC mice orchidectomized 5 and 8 weeks after the transfer of FLC.

EAO could not be induced in the transplanted testis of orchidectomized SCID mice without the transfer of FLC.

Presence of testicular tissue during the early stage of immunological development suppresses induction of EAO in testis transplanted in orchidectomized SCID-FLC mice

Results from the previous experiment suggested that absence of tissue antigens during the early stage of immunological development enhanced the degree of EAO in transplanted testis of SCID-FLC mice. To confirm these results, a different approach was undertaken in the second experiment (Fig. 5). Orchidectomy was first conducted in normal adult SCID mice and then the testis was transplanted 0, 1, 2 or 9–11 weeks after the transfer of FLC. After the full development of the immune system, the SCID-FLC mice were immunized with testicular antigens for the induction of EAO as above. As controls, normal BALB/c mice and untreated SCID mice were first subjected to orchidectomy, then testicular transplantation. Induction of EAO was tested in all these groups and compared in terms of grade of inflammation (Fig. 6).

As expected, absence of testicular antigens during the early phase of immunological development enhanced the degree of EAO in testis transplanted in to SCID-FLC mice 9–11 weeks after the transfer of FLC (grade: 2·7 ± 0·4). As compared with this group, the grade of EAO was significantly reduced in the groups in which the testis had been transplanted 0–2 weeks after the transfer of FLC (Fig. 6). The results in this experiment suggested that presence of testicular antigens in the early developmental phase of the immune system promoted proper establishment of self-tolerance and accordingly ameliorated the grade of EAO in these mice.

DISCUSSION

In the PB of SCID-FLC mice, surface immunoglobulin$^+$ cells started to appear 2 weeks after the transfer of FLC, and their numbers reached a plateau at a level normal for adult BALB/c

Table 1. Induction of EAO in the original or transplanted testes of SCID, SCID-FLC and BALB/c mice

Immunization	mice	*n*	testis	Grade of EAO	means ± SE
Testis + IFA*	SCID-FLC	4	Original	0, 0, 0, 0	0·0
Testis + CFA†	SCID-FLC	4	Original	0, 0, 0, 0	0·0
Testis + CFA†	SCID-FLC	3	Transp.¶	0, 0, 0	0·0
Testis + CFA†	BALB/c	8	Transp.¶	0, 0, 0, 0, 0, 0, 0, 0	0·0
Testis + CFA + BP‡	SCID-FLC	8	Original	3, 5, 5, 1, 5, 2, 5, 1	3·4 ± 0·7
Testis + CFA + BP‡	BALB/c	9	Original	1, 2, 3, 3, 5, 2, 4, 1, 2	2·6 ± 0·4
Testis + CFA + BP‡	SCID-FLC	15	Transp.¶	1, 2, 2, 1, 1, 1, 1, 2, 1, 1, 1, 1, 2, 1	1·3 ± 0·1
Testis + CFA + BP‡	BALB/c	12	Transp.¶	1, 1, 2, 1, 1, 1, 1, 2, 1, 2, 1, 1	1·3 ± 0·1
Testis + CFA + BP‡	SCID	4	Original	0, 0, 0, 0	0·0
Testis + CFA + BP‡	SCID	5	Transp.¶	0, 0, 0, 0, 0	0·0
Saline + CFA + BP§	SCID-FLC	6	Original	0, 0, 0, 0, 0, 0	0·0

SCID-FLC mice were subcutaneously immunized with testicular tissue from weeks 12 to 16 after the transfer of FLC from BALB/c mice. Original and transplanted testes were histologically examined 3 weeks after the immunization.

*Mice were immunized with testicular antigens with IFA.

†Mice were immunized with testicular antigens and CFA.

‡Mice were immunized with testicular antigens and CFA followed by i.v. injection of BP.

§Mice were immunized with saline and CFA followed by i.v. injection of BP.

¶The testis transplanted under renal capsule of mice was examined in terms of EAO induction.

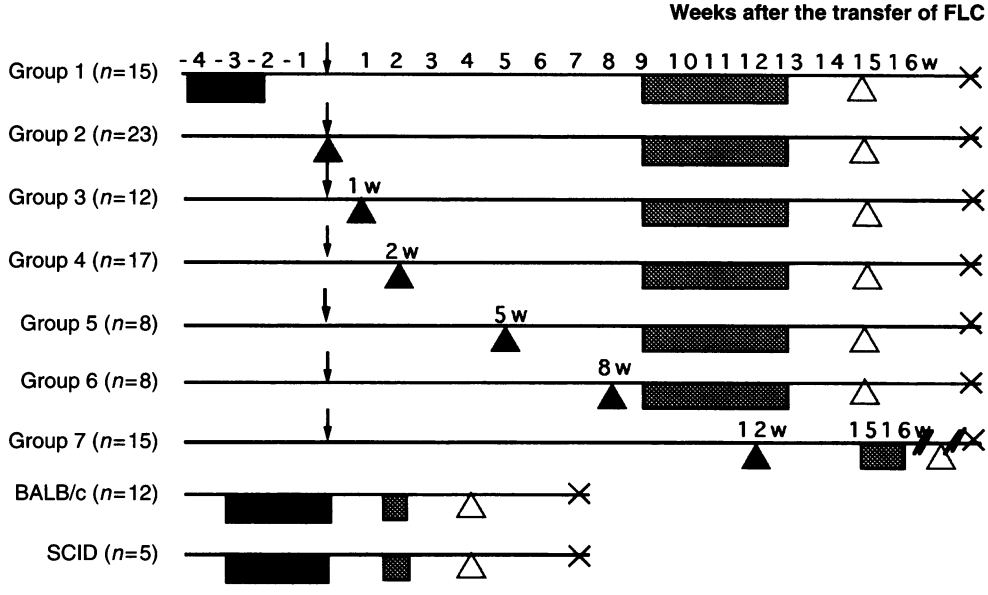

Figure 3. Experimental design of induction of EAO in SCID-FLC mice orchidectomized during development of the immune system. SCID mice were orchidectomized 2–4 weeks before or 0, 1, 2, 5, 8 and 12 weeks after the transfer of FLC. From weeks 9–13 after the transfer, transplantation of testicular tissue from BALB/c mice was carried out in these orchidectomized SCID-FLC mice. In a group of SCID-FLC mice orchidectomized 12 weeks after the transfer, the testis was transplanted 15–16 weeks after the transfer. The transplanted testis was maintained for a period of more than 3 weeks before the induction of EAO. As controls, previously orchidectomized adult BALB/c and SCID mice received testicular transplantation and were immunized 2 weeks later. (↓) indicate i.v. injection of 1×10^7 FLC from BALB/c mice on GD 14. (▲) indicates orchidectomy at this point, and (■) indicates orchidectomy within this term. (▩) indicate the transplantation of the testis from 1-week-old BALB/c mice under renal capsule within this term. (△) indicate the immunization of the testis with CFA and BP, 2 weeks after the transplantation. (×) indicates histological analysis of the transplanted testis 3 weeks after the immunization.

mice in 5 weeks. In contrast, T cells were not detectable until 4 weeks, increasing in number at 5 weeks before levelling off at 8 or 9 weeks after the transfer. It is important to note that the time course of the development of T and B cells in SCID mice immunologically reconstituted with FLC was comparable to that normally observed during the fetal stage. Namely, the pattern of appearance of T cells in PB suggested that the term between 0 and 4 weeks after the transfer of FLC could be comparable to the fetal stage and the term after 4 weeks to the stage after the birth (Fig. 2). A longer duration for immunological development in SCID-FLC mice was convenient for manipulation or analysis of developing immune system in the present study.

When immunization of testicular antigens was

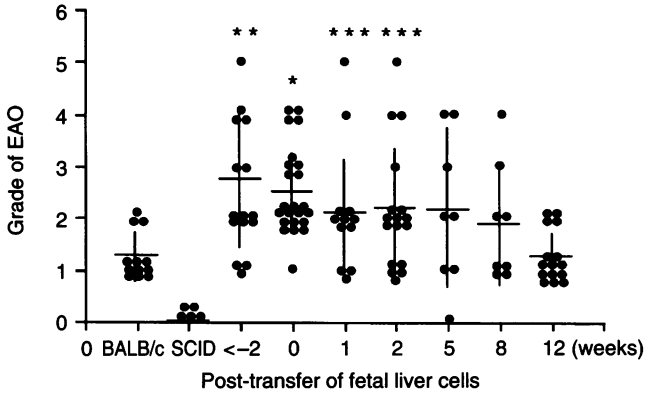

Figure 4. Grade of EAO in the transplanted testis of SCID-FLC mice orchidectomized at various intervals after the transfer of FLC. The number of weeks after i.v. injection of the FLC is shown in the abscissa. The ordinate shows grade of inflammation in the transplanted testis. (●) shows level of each mouse. Horizontal line indicates the mean of each group and vertical line, range of SEM. Statistical significance is shown as *($P<0{\cdot}001$), **($P<0{\cdot}01$) and ***($P<0{\cdot}05$), as compared with the value of SCID-FLC mice orchidectomized 12 weeks after the transfer of FLC (Student's *t*-test).

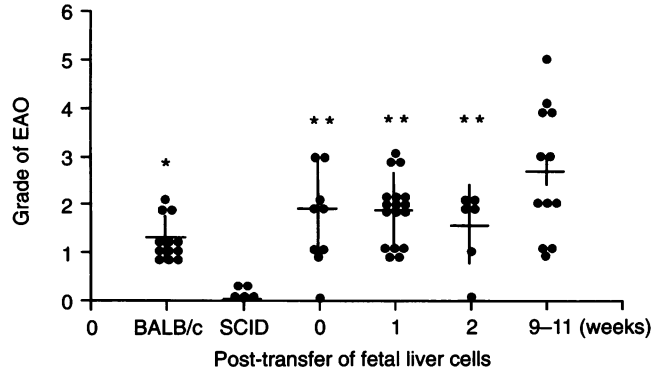

Figure 6. Grade of EAO in orchidectomized SCID-FLC mice that received testicular transplantation at various intervals after the transfer of FLC. The number of weeks after i.v. injection of the FLC is shown in the abscissa. The ordinate shows grade of inflammation in the transplanted testis. (●) shows level in individual mice. Horizontal line indicates the mean of each group and vertical line, range of SEM. Statistical significance is shown as *($P<0{\cdot}01$) and **($P<0{\cdot}05$), as compared with the value of SCID-FLC mice transplanted with testis 9–11 weeks after the transfer of FLC (Student's *t*-test).

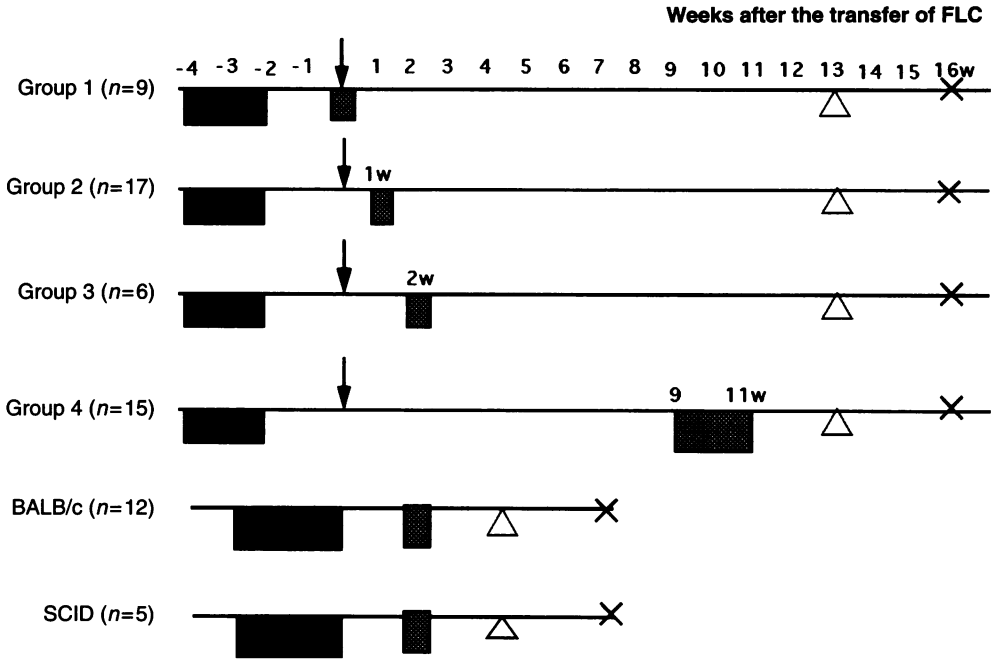

Figure 5. Experimental design of induction of EAO in SCID-FLC mice subjected to orchidectomy and testicular transplantation during development of the immune system. Orchidectomy was first conducted in normal adult SCID mice and then the testis was transplanted 0, 1, 2 or 9–11 weeks after the transfer of FLC. After the full development of the immune system, SCID-FLC mice were immunized with testicular antigens for the induction of EAO. As controls, normal BALB/c mice and untreated SCID mice were first orchidectomized, then given a testicular transplantation. Downward arrows (↓) indicate i.v. injection of 1×10^7 FLC from BALB/c mice on GD 14. (■) indicates orchidectomy within this term. (▩) indicates the transplantation of the testis from 1-week-old BALB/c mice under renal capsule within this term. (△) indicate the immunization of the testis with CFA and BP (×) indicates histological analysis of the transplanted testis 3 weeks after the immunization.

accompanied with that of strong adjuvants (CFA and BP), the degree to which EAO was induced in the testis transplanted under the renal capsule was the same in BALB/c and SCID-FLC mice. In the present study, two experiments were designed for modifying the *in vivo* conditions of contact between the cells of the developing immune system and self antigens in SCID-FLC mice. Firstly, the presentation of the testicular antigens was disturbed by orchidectomy at various points during the development of the immune system in SCID-FLC mice. Secondly, the testicular antigens were exposed to the

cells of the developing immune system at various points by testicular transplantation in previously orchidectomized SCID mice.

In the first experiment, the induction of EAO was accelerated in groups of SCID-FLC mice orchidectomized earlier than 2 weeks after the transfer of FLC when no T cells and few B cells were present in the PB. In contrast, the induction of EAO was suppressed in the testis transplanted in SCID-FLC earlier than 2 weeks after the transfer of FLC in the second experiment. Therefore, exposure of antigens during the early phase of the development of the immune system would be essential for reducing the severity of EAO. This finding is consistent with reports by Triplett,[4] and Eishi and McCullagh.[5] In other words, self-tolerance can be established by contact between self-tissue antigens and immune cells in the early developmental stage in SCID-FLC mice.

In transgenic mice expressing simian virus 40 (SV40) T antigen in pancreatic islets alone, the production of autoantibody to SV40 T antigen and the disruption of the pancreatic islets were observed in those mice in which SV40 T antigen expresses after the adult stage, but not in those in which the antigen expresses before the neonatal stage.[7]

Akashi and Eishi[8] reported that autoantibodies were never produced against tissue antigens expressed before the development of CD4 and CD8 T cells. These results support our finding that the induction of EAO was suppressed in the presence of self-antigens during the early stage of immunological development.

The results in the present study indicate that self-tolerance to tissue antigens occurs in the early stage of immunological development. Such tolerance might be ascribed to one or all of the following: deletion of autoreactive T cell probably in the thymus; anergy of autoreactive T cells by an unknown mechanism; generation of regulatory T cells.

It was reported that potentially autoreactive T cells specific to organs are present in normal healthy mice, and the organ-specific autoimmune disease could be induced by depletion of particular T-cell subsets, which probably suppressed the autoreactive T cells.[9,10,11] Recently, the involvement of regulatory T cells was implicated in a study using a transgenic mouse strain expressing a T cell receptor specific for the self myelin basic protein.[12] Also, it was suggested that injection of lymphocytes sensitized with thyrogloblin into neonatal rat induced resistance against experimental autoimmune thyroiditis after maturation of the recipient rats.[13]

It is likely that acquisition and subsequent maintenance of self-tolerance is impaired, when the developing immune system is not effectively exposed to the organ-specific self antigens in the periphery outside the thymus during a critical period of the development. Thus, microorganisms (e.g. of bacterial and viral origin) may stimulate autoreactive clones that failed to be eliminated by mechanisms of central and peripheral deletion, resulting in the induction of organ-specific autoimmune disease.

At any event, the results in the present study suggest that immunologically reconstituted SCID mice (SCID-FLC mice) can be employed as an experimental model of developing immune system and used for the precise analysis of the process of self-tolerance. Studies are now in progress to elucidate whether cells of the immune system are involved in the regulation of the induction of EAO in SCID-FLC mice.

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