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Experimental autoimmune orchitis after neonatal thymectomy in the mouse

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SUMMARY

Experimental autoimmune orchitis (EAO) developed in (C57Bl/6Cr × A/JCr)F_1 mice 3–4 months after neonatal thymectomy (Tx) without any sensitization. The age of Tx was critical for induction of EAO: Tx at day 3 (Tx-3) was effective, but Tx at day 0 or day 7 was not effective. This lesion resulted in atrophy of the testis and was characterized by disappearance of mature sperms, formation of multinuclear giant cells in seminiferous tubules and infiltration of lymphocytes in the stroma. Epididymitis was observed prior to the development of EAO. Presence of circulating autoantibody(s) against sperms (ASA) was demonstrated by indirect immunofluorescence. The acrosomal area of mature sperms, but not of immature spermatids, was stained strongly. The incidence of EAO and titre of ASA increased when Tx-3 mice were unilaterally vasectomized (Vx). The majority of mice with high titres of circulating ASA were sterile. Epididymitis and orchitis could be prevented in Tx-3 mice by injection of adult spleen cells on day 4. The most effective source was normal male. Spleen cells from normal female donors and day-0 orchidectomized (Orx) donors were less effective, while those of Tx-3 male and female donors failed to prevent epididymitis and orchitis. The cell population in normal male spleen effective in preventing epididymitis was shown to be a T cell population (Thy 1^+, Ig^-) by experiments with respective antisera treatment. These results showed that sensitization with sperm autoantigen occurred in the epdidymis after Tx-3, more efficiently after Tx-3 plus unilateral Vx, and that this autosensitization was prevented by a specific suppressor T cell population, which was present in normal males but absent in Tx-3 mice.

INTRODUCTION

Recently, we reported that experimental disturbance of immunological regulation induced by neonatal thymectomy (Tx) in the mouse without any additional treatment causes organ-localized autoimmune lesions, such as thyroiditis (Kojima *et al.*, 1976a), gastritis (Kojima, Taguchi & Nishizuka, 1980) and oöphoritis (Taguchi *et al.*, 1980). In this system, the age of mice at the time of Tx is critical for the induction of autoimmune diseases. The most effective days of Tx are days 2–4 (the day of birth is day 0), while Tx before or after this critical age is not effective. These diseases can be prevented by inoculation of T cells from syngeneic adult mice (Sakakura & Nishizuka, 1972; Kojima *et al.*, 1976b). In this paper we report our results on autoimmune orchitis developed in neonatally Tx mice. Since in humans (Ansbacher, 1971; Samuel *et al.*, 1975) and in experimental animals (Rumke & Titus, 1970; Bigazzi *et al.*, 1976; Alevander & Tung, 1977), long-term vasectomy (Vx) has been shown to result in an autoimmune response to sperms, we have also attempted to investigate the effect of unilateral Vx in these mice. The possible mechanism of self-recognition and autoimmunity in this system is discussed.

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MATERIALS AND METHODS

Animals. Hybrids of (C57Bl/6Cr × A/JCr)F_1 (B6A) mice were used in these experiments as described previously (Taguchi & Nishizuka, 1980).

Thymectomy (Tx), orchidectomy (Orx) and unilateral vasectomy (Vx). Tx was performed on day 0 (within 12 hr after birth), day 3 or day 7 (Tx-0, Tx-3, Tx-7) by the technique described previously (Nishizuka & Sakakura, 1971). Orx was performed on day 0 under ether anaesthesia. Unilateral Vx was performed at the site of the left ductus deferens (DD) or ductuli efferentes (DE) under ether anaesthesia on day 60. All the mice were killed at the age of 30–150 days. At autopsy thymic remnants were carefully checked in all the mice.

Preparations of spleen cell suspensions. Cell suspensions were prepared, as previously described (Taguchi & Nishizuka, 1980) from two or four fresh spleens obtained from 4-month-old normal male, normal female or Orx mice and were adjusted to final concentrations of 4×10^5, 4×10^6 and 4×10^7/0·1 ml. To exclude T cells or B cells from the inocula, the spleen cell suspensions prepared from normal male mice were incubated with anti-Thy 1.2 antiserum (Searle Diagnostic, UK, dilution 1:20) or with anti-Ig antiserum (MBL Ltd, Japan, dilution 1:20) plus guinea-pig complement (GPC) dilution (1:5) as described previously (Taguchi & Nishizuka, 1980). Final cell concentrations were adjusted to 4×10^7/0·1 ml.

Spleen cell injections. Syngeneic Tx-3 mice received a single i.p. injection of 0·1 ml spleen cells suspension at day 4, and recipients were killed at 90 days of age.

Histology. All mice were autopsied. Tissues were fixed in Bouin's fixative, embedded in paraffin, sectioned and stained with haematoxylin and eosin for histological observation.

Immunohistology. The testis and epididymis of one side of 2-month-old and 5-month-old Tx-3 and control mice were sectioned by cryostat and fixed with 95% ethanol. The testis and epididymis of the other side were fixed with Bouin's fixative for histological observation. The cryostat sections were incubated with FITC-labelled anti-mouse IgA, IgG, IgM and C3 for 30 min at 37°C, and washed in PBS for 15 min for immunohistological observation.

Collection of sera. Mice were exsanguinated through the axillary artery under ether, and sera from individual mice were stored at −80°C until used.

Indirect immunofluorescence (IFL) technique. Cryostat tissue sections of 1-month-old testes, 3-month-old testes and epididymis, and sperm smears of normal B6A mice were fixed with 95% ethanol, and used for IFL studies by the technique described previously (Taguchi *et al.*, 1980). Sperms were obtained by cutting up the epididymis in PBS and filtering through a stainless mesh sieve to remove tissue fragments; then washed twice with PBS by centrifugation at 900 r.p.m. for 5 min at 4°C.

Specificity test. To test the specificity of positive IFL reactions, absorption tests were carried out by the method described previously (Taguchi *et al.*, 1980). Tissue homogenates used for absorption were prepared from intact 1-month-old or 3-month-old testes of B6A mice, and from a Leydig cell tumour induced by intra-splenic grafting of a testis in a B6A mouse (Taguchi, unpublished).

Fertility test. To investigate reproductive capacity, groups of mice that had undergone Tx, unilateral Vx alone, Tx plus unilateral Vx and control mice, were mated on day 120 with two normal females for a period of 14 days. When one or two partners became pregnant, male mice were regarded as having fertile capacity.

RESULTS

Pathology of orchitis and epididymitis induced by neonatal Tx. Orchitis induced by Tx-3 without any sensitization with testicular antigen is characterized by disturbances of spermatogenesis with appearance of multinuclear giant cells and lymphocyte infiltration in the stroma (Fig. 1). In severe cases, complete loss of germ cells was observed and seminiferous tubules contained only Sertoli cells. Such orchitis was first seen histologically at 3–4 months of age (Table 1). Probably, prior to the development of orchitis, a lesion occurred in the epididymis at around 40 days of age. This was

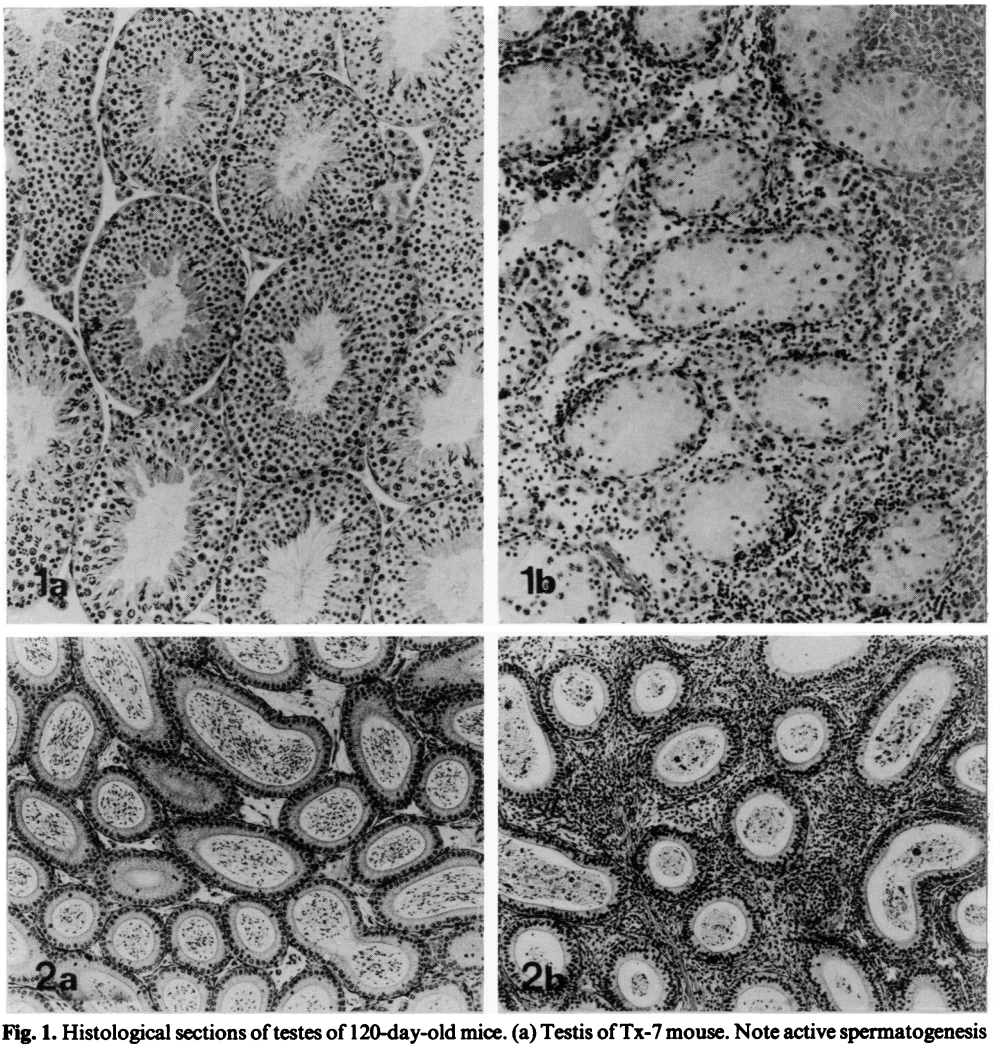


Fig. 1. Histological sections of testes of 120-day-old mice. (a) Testis of Tx-7 mouse. Note active spermatogenesis in seminiferous tubules. (b) Typical pattern of orchitis of Tx-3 mouse. Note aspermatogenesis associated with lymphocyte infiltration in stromal tissues. (Original × 130.)

Fig. 2. Histological sections of epididymis of 60-day-old mice. (a) Epididymis of Tx-7 mouse. Note many normal sperm in ducts. (b) Epididymis of Tx-3 mouse. Note degenerated sperm in ducts and infiltration of lymphocytes and granulocytes in the stroma. (Original × 75.)

manifested by infiltration of lymphocytes and granulocytes in the subepithelial tissues and accumulation of degenerated sperms in epididymal ducts (Fig. 2).

Effect of age of Tx on development of epididymitis and orchitis. Data shown in Table 1 clearly indicate that orchitis and epididymitis occurred in mice receiving Tx-3, but not in mice receiving Tx-0 and Tx-7.

Detection of circulating autoantibody against sperm (ASA). Circulating ASA was detected in sera of mice with orchitis and/or epididymitis by IFL technique (Table 1). Fluorescence was confined to acrosomes of mature sperms but was not seen in spermatids in seminiferous tubules of adult testes (Fig. 3). Sections of immature testes of 1-month-old mice did not give positive IFL reaction. Sperms in smear preparations obtained from the adult epididymis also gave positive IFL reaction (Fig. 4). Correlation of ASA with histological features of epididymitis and orchitis are

Table 1. Effect of neonatal thymectomy on induction of epididymitis and orchitis

Age at thymectomy (days)	Day killed	No. of mice: Total	With epididymitis (%)	With orchitis (%)	With antibody against sperm* (%)
—	30–150	90†	0 (0)	0 (0)	0 (0)
0	120	10	0 (0)	0 (0)	0 (0)
3	30	10	0 (0)	0 (0)	0 (0)
	40	11	4 (36)	0 (0)	0 (0)
	50	13	8 (62)	0 (0)	0 (0)
	60	15	7 (47)	0 (0)	2 (13)
	90	22	9 (41)	1 (4·5)	4 (18)
	120	22	10 (45)	5 (23)	9 (41)
	150	24	12 (50)	4 (17)	10 (42)
7	60	20	0 (0)	0 (0)	0 (0)
	150	23	0 (0)	0 (0)	0 (0)

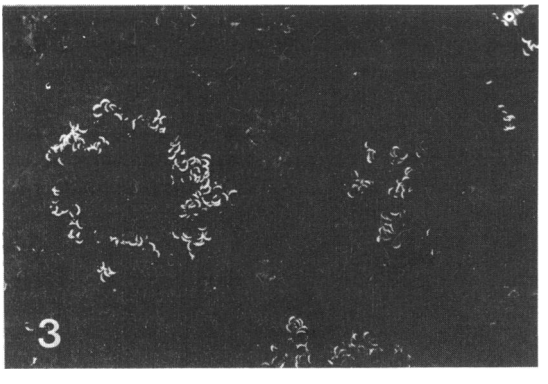
* Antibody was detected by IFL reaction with testis sections.

† 10–15 mice were used in all groups killed at 30, 40, 50, 60, 120 and 150 days of age.

shown in Fig. 5. A good correlation was observed between ASA and lesions: ASA with high titres appeared at 120–150 days of age when severe epididymitis and orchitis was found.

To test the specificity of the anti-sperm IFL reaction, sera giving positive IFL reactions were absorbed by homogenates of adult testes. Such sera did not give a positive IFL reaction. Absorption with homogenates of 1-month-old immature testes and of tissues of Leydig cell tumour did not affect the IFL reaction against mature sperm.

Immunohistological findings. When cryostat sections of the testis and epididymis of Tx-3 mice were incubated with FITC-labelled anti-mouse IgA, IgG, IgM and C3, granular deposits of IgA, IgG, IgM and C3 were detected around the basement membrane of the seminiferous tubules of the testis (Fig. 6) and the epididymal ducts (Fig. 7). Table 2 correlates immunohistological findings with the presence of orchitis and epididymitis. Deposits of IgG were most frequent and abundant among



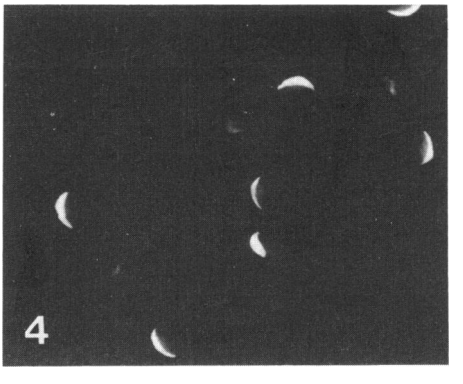


Fig. 3. Cryostat section of testis of normal 90-day-old mouse incubated with serum (1:160 dilution) of Tx-3 mouse (day 120) followed by FITC-labelled anti-mouse IgG. Note fluorescence of mature sperms. (Original × 160.)

Fig. 4. Normal sperm in smear preparation incubated with serum (1:160 dilution) of Tx-3 mouse (day 120) followed by FITC-labelled anti-mouse IgG. Note fluorescence in the acrosomal area. (Original × 400.)

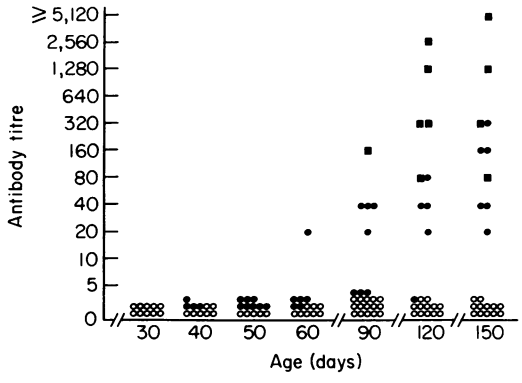


Fig. 5. Correlation of antibody against sperm with histology of epididymis and testis. (o) Mice with normal epididymis and testis; (•) mice with epididymitis and normal testis; (■) mice with epididymitis and orchitis.

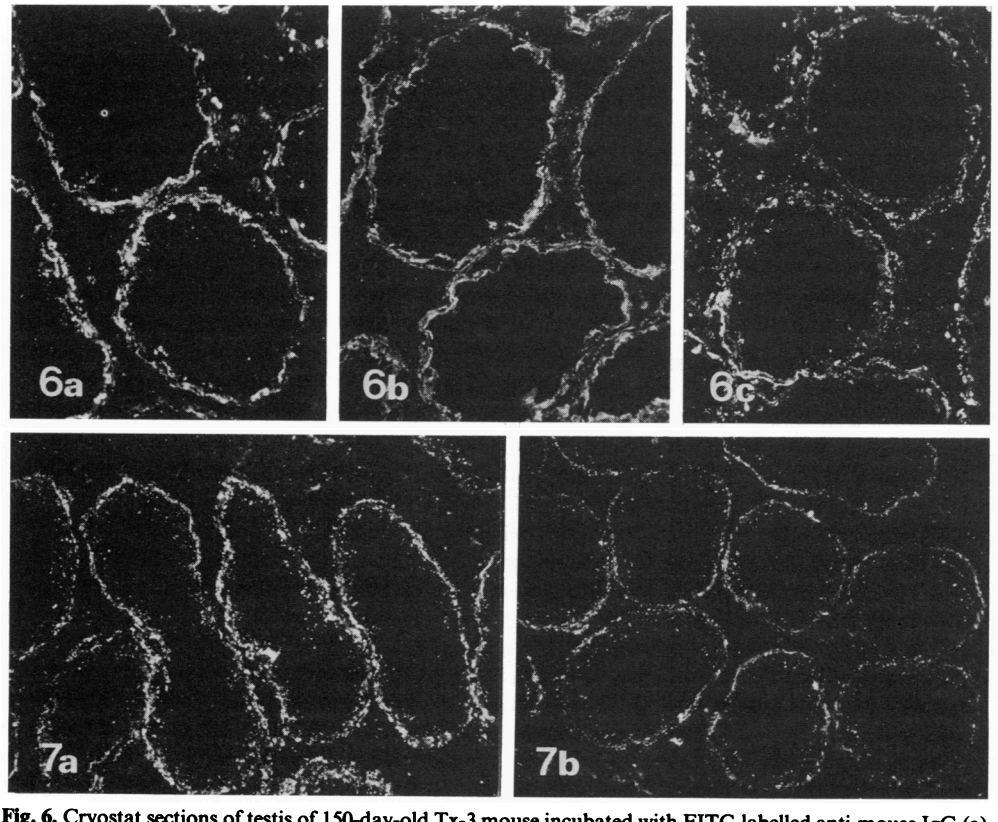


Fig. 6. Cryostat sections of testis of 150-day-old Tx-3 mouse incubated with FITC-labelled anti-mouse IgG (a) IgM (b), and C3 (c). Note granular deposits of immunoglobulins and C3 around the basement membrane of seminiferous tubules. (Original × 160.)

Fig. 7. Cryostat sections of epididymis of 150-day-old Tx-3 mouse incubated with FITC-labelled anti-mouse IgG (a) and C3 (b). Note granular deposits of immunoglobulins and C3 around the basement membrane of epididymal ducts. (Original × 160.)

Table 2. Immunohistological findings of the testis and epididymis*

Age at thymectomy (days)	Day killed	Mouse No.	Epididymitis	Orchitis	Epididymis. Deposite of: IgA	IgG	IgM	C3	Testis. Deposit of: IgA	IgG	IgM	C3	Titre of anti-sperm antibody
—	60	1–7	−	−	−	−	−	−	−	−	−	−	0
—	150	1–7	−	−	−	−	−	−	−	−	−	−	0
3	60	1	+	−	−	+	−	+	−	−	−	−	0
		2	+	−	−	−	+	−	−	−	−	−	0
		3	+	−	−	−	+	−	−	−	−	−	0
		4	+	−	−	−	−	−	−	−	−	−	0
		5	−	−	−	−	−	−	−	−	−	−	0
		6	−	−	−	−	−	−	−	−	−	−	0
3	150	1	+	+	+	+	+	+	+	+	+	+	1,280
		2	+	+	−	+	+	+	+	+	+	+	80
		3	+	+	−	+	+	+	−	+	+	+	5,120
		4	+	−	+	+	+	+	−	+	−	−	320
		5	+	−	−	+	+	+	−	−	−	−	160
		6	+	−	−	+	+	+	−	−	−	−	40
		7	+	−	−	+	−	+	−	−	−	−	20
		8	−	−	−	−	−	−	−	−	−	−	0
		9	−	−	−	−	−	−	−	−	−	−	0
		10	−	−	−	−	−	−	−	−	−	−	0
7	60	1–6	−	−	−	−	−	−	−	−	−	−	0
7	150	1–7	−	−	−	−	−	−	−	−	−	−	0

* The testis and epididymis of one side were used for immunofluorescence staining and those of the other side for histological observation by haematoxylin and eosin staining.

deposits of immunologlobulins and C3. In 150-day-old Tx-3 mice, positive correlation was also observed between the deposits of immunologlobulins and detection of ASA. However, in 60-day-old Tx-3 mice, no circulating ASA were detected even though deposits of immunogloblins were observed in the epididymal ducts. No immunogloblins, C3 deposits, or inflammatory reactions were observed in the testis and epididymis of intact and Tx-7 mice; also, no antibody was detected.

Effect of injection of spleen cell suspension. To prevent the epididymitis which developed prior to EAO in Tx-3 mice, a single i.p. injection of different doses of spleen cells obtained from various donors was performed on day 4. The development of epididymitis was prevented by injections of spleen cells obtained from normal male, normal female and Orx male donors (Table 3). No testicular changes were found in the recipients up to 150 days old. Interestingly, the effective dose of spleen cells varied according to the donor, i.e. the minimal effective dose from male donors was 4×10^6, while that from female and Orx donors was 4×10^7. In contrast, injection of spleen cells (4×10^7) obtained from Tx-3 male and female donors failed to prevent the epididymitis, and a higher incidence of epididymitis and earlier development of orchitis were observed in these recipients. Pretreatment of spleen cells prepared from normal male donors with anti-Thy 1.2 antiserum plus GPC abolished the ability to prevent epididymitis when injected i.p. into Tx-3 mice on day 4. However, spleen cells pretreated with anti-Ig antiserum plus GPC still maintained the ability to prevent the lesion.

Effect of unilateral Vx. Unilateral Vx at the sites of DD or DE enhanced both incidence and severity of the orchitis in Tx-3 mice (Table 4). Left testes which received excision of excretory duct showed a higher incidence of orchitis. The detection rate of circulating ASA was also increased in both groups. It appeared that Vx at DD was more effective than Vx at DE in terms of the development of orchitis. Also, higher levels of ASA were detected in the Tx-3 plus DD-Vx group

Table 3. Effect of injecting different doses of spleen cells from different donors on prevention of epididymitis*

Cell source	No. of cells injected	No. of mice examined	No. of mice with epididymitis (%)	Effects on prevention
—	—	22	9 (41)	—
Normal male	4×10^5	12	6 (50)	Neg
	4×10^6	26	2 (8)	Pos
	4×10^6	22†	1 (5)	Pos
	4×10^7	14	0 (0)	Pos
Normal female	4×10^5	14	5 (36)	Neg
	4×10^6	16	6 (38)	Neg
	4×10^7	19	0 (0)	Pos
Orx male	4×10^6	24	11 (46)	Neg
	4×10^7	15	0 (0)	Pos
Tx-3 male	4×10^7	16‡	11 (69)	Neg
Tx-3 female	4×10^7	12‡	8 (67)	Neg

* Spleen cells obtained from 4-month-old donors were injected i.p. into Tx-3 mice at day 4, and the recipients were killed at 90 days of age.

† Recipients were killed at 150 days of age and histology of the testes of all mice was normal.

‡ Orchitis was observed in five male (31%) and four female (33%) recipients.

than in the Tx-3 plus DE-Vx group (Fig. 8). In contrast, active spermatogenesis was observed in both DD-Vx and DE-Vx only groups. Circulating ASA were detectable in a few DD-VX mice, but their titres were very low. No circulating ASA were detected in the mice of the DE-Vx group.

Reproductive capacity. Fertility testing showed that male reproductive performance was disturbed by neonatal Tx (Table 4). The fertility of Tx-3 mice was further reduced after unilateral Vx. Some sterile mice had high titres of circulating ASA (Fig. 8). In contrast, unilateral DD-Vx or DE-Vx in non-Tx mice did not disturb the reproductive capacity even though the mice had circulating ASA.

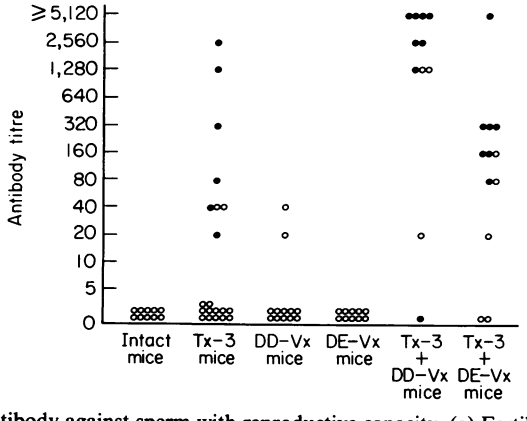


Fig. 8. Correlation of antibody against sperm with reproductive capacity. (o) Fertile mice; (•) sterile mice.

Table 4. Effect of neonatal thymectomy and unilateral vasectomy on development of orchitis and on reproductive performance*

Group	No. of mice used	No. of mice with orchitis		No. of mice with anti-sperm antibody (%)	No. of sterile mice (%)
		Left testis (%)	Both testes (%)		
Intact	10	0 (0)	0 (0)	0 (0)	0 (0)
Tx-3	20	5 (25)	5 (25)	8 (40)	6 (30)
DD-Vx	12	0 (0)	0 (0)	2 (17)	0 (0)
DE-Vx	10	0 (0)	0 (0)	0 (0)	0 (0)
Tx-3+DD-Vx	11	10 (91)	5 (45)	10 (91)	8 (73)
Tx-3+DE-Vx	12	11 (92)	3 (25)	10 (83)	7 (58)

* Unilateral Vx was performed at the left excretory duct (see Materials and Methods).

DISCUSSION

It is widely accepted that immunization with homogenates of antigenic materials of the testis in Freund's adjuvant causes experimental allergic autoimmune orchitis in a variety of animals (for review *see* Shulman, 1971). These experiments are useful models for studying the mechanisms of testis-specific autoimmune disease, especially for analysis of antigens (Brown, Holborow & Glynn, 1965; Voisin & Toullet, 1968; Toullet, Voisin & Nemirovsky, 1973; Hagopian *et al.*, 1975). However, many questions still remained in these models in relation to immunological status of host animals. The present experiments have demonstrated that neonatal Tx in the mouse provides a new model for EAO. The autoimmune nature of this model was strongly supported by the consistent appearance of specific antibody against sperm, particularly acrosomes, in sera of mice with orchitis. Profound disturbance of spermatogenesis with subsequent sterility and remarkable accumulation of lymphocytes in the peritubular interstitium were striking features of the orchitis. About 2 months prior to the development of EAO which developed at about 4 months of age, inflammatory lesions characterized by lymphocytic and granulocytic infiltration appeared in the epididymis. At this stage, sera of mice with epididymitis had no detectable circulating antibody against epididymal tissues. Hence, the inflammatory reaction of epididymis was a response to the sperms, and granular deposits of immunoglobulins and C3 along the basement membranes of epididymal ducts and seminiferous tubules may be immune complexes. Circulating ASA were detected in sera of mice at 60–90 days of age when a severe inflammatory reaction was usually observed in the epididymis. Subsequently, good correlation of antibody titres with severity of lesions in epididymis and testis was noted. Significantly, positive IFL staining of sperm acrosomes was obtained with sections of the mature testis but not of immature testis. This indicates perhaps that new antigens appear in acrosomes as germ cell progeny mature. This is also the case in post-thymectomy autoimmune oöphoritis where antigen appears with the process of ripening of oöcytes (Taguchi *et al.*, 1980).

It has been reported that with Vx at DD, millions of spermatozoa remain within the epididymis when sperm antigens may be released into the circulation and elicit anti-sperm antibodies (Rumke & Titus, 1970). In the present experiment, 10 weeks after unilateral Vx, circulating ASA of low titres were detected in a few mice of DD-Vx groups, while no ASA were detected in all mice of the DE-Vx group; in both groups, no inflammatory reaction was observed, however. Probably, this is connected with the short-term observations of our system compared with other experimental systems of Vx with rats (Rumke & Titus, 1970), rabbits (Bigazzi *et al.*, 1976; Alevander & Tung, 1977), rats and mice (Kosuda & Bigazzi, 1979). Autoimmune orchitis and ASA were more frequently detected in Tx-3 plus Vx groups than in Vx and Tx groups. As to the site of Vx, excision at

DD was more effective than at DE. It has been reported that the epididymal duct may be a site of absorption of fluid materials produced in the testis (Hamilton, 1972) and this data may indicate that exposure to sperm antigens was enhanced by Vx, and the self-recognition mechanism was disturbed by neonatal Tx. The finding that severe testicular damage is frequently observed at the left testis which received Vx compared with that in the right testis which did not receive Vx supports this idea, and perhaps is connected with some disorders of the testis–blood barrier (Johnson & Setchell, 1968; Vitale, Fawcett & Dym, 1973).

A hypothesis to explain the mechanism of autoimmune diseases induced by Tx-3 but not Tx-0 or Tx-7 has been described before (Taguchi *et al.*, 1980; Taguchi & Nishizuka, 1980). Namely, in Tx-3 male mice, some T cells are specifically autoreactive to testicular antigens. In normal and Tx-7 male mice, there exist specific suppressor T cells, which specifically inhibit activation of these autoreactive T cells. Neither of these T cell populations are present in Tx-0 mice. The possible existence of suppressor T cells in normal mice but not in Tx-3 mice is supported by the results of injecting spleen cells from syngeneic adult donors into Tx-3 recipients. In preventing developments of autoimmunity, the spleen cells from normal males were more effective than those of normal females and Orx donors. This suggests that in normal males, specific suppressor T cells for testicular antigens exist by sensitization of autoantigens of the testis, while in normal female and Orx mice such specific suppressor T cells may be absent or less in number. Injection of spleen cells from Tx-3 male and female donors enhanced the incidence of both epididymitis and orchitis in the Tx-3 recipients, suggesting the existence of T cells autoreactive against both male and female germ cells in Tx-3 mice, and of antigen(s) common to both male and female germ cells (Taguchi, Sakakura & Nishizuka, unpublished).

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