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## Chronic Changes of Autoimmune Responses to Testis Material in Male Nile Tilapia, *Oreochromis niloticus*

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### Abstract

Time-course changes of autoimmune responses to testis material in males of the Nile tilapia, *Oreochromis niloticus*, were examined for 8 months following injections of allogeneic testis material emulsified in Freund's complete adjuvant. When maturing males were immunized, high sperm agglutination titers in their sera were detected 1 month after immunization and were retained for at least 7 months. Enlargement of the spleen was also conspicuous in treated fish. By contrast, when mature males were immunized, sperm agglutination titers of the serum reached a peak of a similar level to that found in the maturing males, and then decreased. The agglutination pattern in the serum was of the tangle type for allogeneic spermatozoa, while it was mainly of the head-to-head type for autologous spermatozoa. In testes of the immunized fish, granulomatous structures appeared in seminal lobules and efferent ducts together with immune cells attacking spermatozoa, beginning from 3 months after immunization. These pathological changes occurred only locally in affected testes and appeared to be alleviated considerably as the testes grew to maturity, never preventing spermatogenesis from advancing normally in the affected testes. Discussions were made on the possible interrelation between humoral and cellular immune responses to testis material in the tilapia.

### Introduction

Recent investigations have demonstrated that injection of autologous or allogeneic testis material can elicit autoimmune lesions in the testis of seasonal breeders, the Atlantic salmon *Salmo salar* (Laird et al., 1980) and the rainbow trout *Salmo gairdneri* (Secombes et al., 1984, 1985a), and a continuous breeder, the Nile tilapia *Oreochromis niloticus* (Lou and Takahashi, 1987). It has been shown also that the immunization treatment can effectively induce the appearance of possible antibodies against spermatozoa in the serum of the rainbow trout (Secombes et al., 1982, 1984) and the Nile tilapia (Lou and Takahashi, 1987). These investigations were conducted to develop an immunological means to control sexual maturation in fishes, but failed to completely sterilize the male. It was reported, however, complex giant cell granulomas found within testes of autoimmunized rainbow trout often prevented spermatozoa from being released (Secombes et al., 1985b).

In the present study, the occurrence and maintenance of serum factors with apparent sperm agglutination titers was observed for a long period lasting up to 8 months in adult males of the Nile tilapia, *Oreochromis niloticus*, immunized with injections of allogeneic testis material. Pathological lesions of the testis affected by immunization were also examined histologically, and their alterations during the

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experimental period were considered in relation to changes of sperm agglutination titers of the serum of immunized fish.

## Materials and Methods

### *Fish*

Maturing and mature males of the Nile tilapia, *Oreochromis niloticus*, were used as material in the present study. Maturing males used as recipients of antigens for autoimmunization were grown from fish of a single brood which had received an oral administration of methyltestosterone at a dosage of 50  $\mu\text{g/g}$  diet for 4 weeks beginning from 7 days after hatching. More than 95% of the treated fish were found to be males. They were kept in glass aquaria equipped each with a filtration-circulation apparatus throughout the present study. Mature males used as donors of antigenic sources were obtained from a commercial tilapia farm, and subsequently reared in an indoor concrete pond of our laboratory. Some mature males were used also as test fish for the induction of autoimmunization. All fish were maintained at water temperature of  $22 \pm 2^\circ\text{C}$  under natural light conditions, and fed a commercial pelleted diet for trout culture every morning.

### *Antigens*

Two kinds of cellular material were used as antigens for immunization. (1) Allogeneic testis homogenate (ATH): mature testes from freshly killed tilapia were homogenized at  $3^\circ\text{C}$  in a glass homogenizer with an equal volume of 0.7% saline. (2) Allogeneic spermatozoa (AS): fresh spermatozoa stripped from mature male tilapia were washed three times with 0.7% saline by centrifugation, and adjusted to  $6 \times 10^7$  cells/ml saline. Each fish to be immunized received intraperitoneal or intramuscular injections of either ATH or AS emulsified in an equal volume of Freund's complete adjuvant (FCA) at a dose of 2.5  $\mu\text{l/g}$  body weight.

### *Immunization regime*

Three sets of experiments were carried out in the present study (Table 1). Maturing males in experiments 1 and 2 received two weekly intraperitoneal injections of ATH + FCA and AS + FCA, respectively. In experiment 1, three fish were

Table 1. Experimental designs for long-term observations of autoimmune responses to testis material in the Nile tilapia, *Oreochromis niloticus*.

Experiment No.	Body weight of fish (g)	Number of males treated	Material injected	Number of injections (route)
1	$36.75 \pm 6.50$	19	FCA	2(i.p.)
	$37.03 \pm 6.83$	23	ATH + FCA	
2	$19.79 \pm 4.37$	10	FCA	2(i.p.)
	$18.76 \pm 4.34$	10	AS + FCA	
3	150-200	2	FCA	3(i.m.)
	150-200	4	ATH + FCA	

bled and sampled every month for 7 months beginning from 1 month after the first injection. The remaining two fish were kept alive following a blood sampling at the 8th month. In experiment 2, three fish each were bled and sacrificed at intervals of 2 months beginning from 3 months after the first injection. In addition, in experiment 3, four mature males were immunized with three weekly intramuscular injections of ATH+FCA and bled, as a rule, 4, 6, 8, 10, and finally 20 weeks after the first injection. In each experiment, fish injected with FCA+saline served as controls.

#### *Antibody titration*

Blood samples were collected in non-heparinized syringes from the caudal vasculature of fish anesthetized lightly with ethyl 4-aminobenzoate and, following centrifugation at 1,500 g for 15 min, sera were obtained and stored at  $-40^{\circ}\text{C}$  until use.

A sperm agglutination test was carried out for sera obtained from both the immunized and control fish according to the method described by Secombes et al. (1982) with a slight modification. All sera were decomplemented at  $50^{\circ}\text{C}$  for 30 min immediately before use. Twenty-five  $\mu\text{l}$  of the sera were serially diluted in Baker's phosphate-buffered saline in microtiter plates. To each well of the plate was added an equal volume of fresh tilapia spermatozoa adjusted to  $2.8\text{--}3.6 \times 10^7$  cells/ml saline. After incubation at room temperature for 3 hr, the microtiter plates were kept at  $4^{\circ}\text{C}$  overnight to allow the spermatozoa to settle. Controls for the agglutination test included both saline and sera of intact tilapia under the same conditions. Allogeneic blood cells were also used for agglutination test as a control experiment. Sperm agglutination titers (SAT) were determined by the formula,  $\text{SAT} = -\log_2 X$ , where  $X$  was the dilution of serum at which sperm agglutination became undetectable. In order to know whether sera from the fish immunized with allogeneic testis material could agglutinate their own spermatozoa, sera from the mature males in experiment 3 were also tested for their SAT using their own spermatozoa. Agglutination patterns of spermatozoa of different origins were compared by phase-contrast microscopy.

#### *Histology*

The testis, liver and spleen of both immunized and control fish in experiments 1 and 2 were fixed in Bouin's fluid for histological examinations. Serial paraffin sections of these organs were cut at  $5 \mu\text{m}$  in thickness and stained with Delafield's hematoxylin and eosin. Stages of testicular maturity were determined according to the criteria proposed by Hyder (1969) for tilapias. For the spleen obtained from the fish in experiment 1, ratios of the area of the largest cross sections of the organ to body weight were calculated and represented as the spleen area-somatic index (SaSI).

## Results

#### *Sperm agglutination titers of the serum*

Serum titers to agglutinate allogeneic spermatozoa were measured for immunized and control fish. Time-course changes of the titers obtained in experiments 1

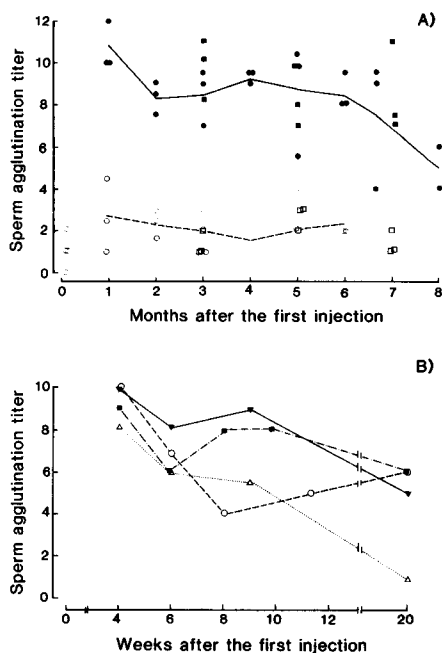


Fig. 1. A) Changes of sperm agglutination titers of sera from maturing male tilapia treated with ATH+FCA (●) or FCA alone (○) in experiment 1, and with AS+FCA (■) or FCA alone (□) in experiment 2. Lines in the figure are connections of mean titers of immunized and control fish in experiment 1.

B) Changes of sperm agglutination titers of sera from four individual mature males (○, ▼, ■, △) treated with ATH+FCA in experiment 3.

and 2 are shown in Fig. 1A. In the fish injected with ATH+FCA in experiment 1, the titers peaked by 1 month after the first injection and remained at a relatively high level until up to 7 months. The serum samples taken 8 months after the first injection gave rather low titers. In immunized fish in experiment 2, the 2-month intervals between each blood sampling rendered the peak of serum titers obscure, but the titers remained at a high level 7 months after the first injection, showing no significant difference from those determined for fish in experiment 1. No agglutination titers were detectable for allogeneic blood cells. Sera of control fish in both experiments showed very low sperm agglutination titers.

Sera from mature males immunized with ATH+FCA in experiment 3 could agglutinate both allogeneic and autologous spermatozoa. The immunized fish exhibited high agglutination titers in their sera 4 weeks after the first injection, followed by a decline in titers continuing to more than 20 weeks after the first injection (Fig. 1B). Different patterns of agglutination were observed for spermatozoa of the different origins. Agglutination occurred along the whole length of allogeneic spermatozoa (Fig. 2a), while it mainly showed a head-to-head pattern for autologous spermatozoa (Fig. 2b).

#### *Spleen area-somatic index (SaSI)*

SaSI values measured for immunized fish in experiment 1 were larger than those for control fish, being four times larger than control values during the first 3 months after the first injection. The values tended to decrease slowly thereafter, though they were still higher than the control 6 months after injection (Fig. 3).

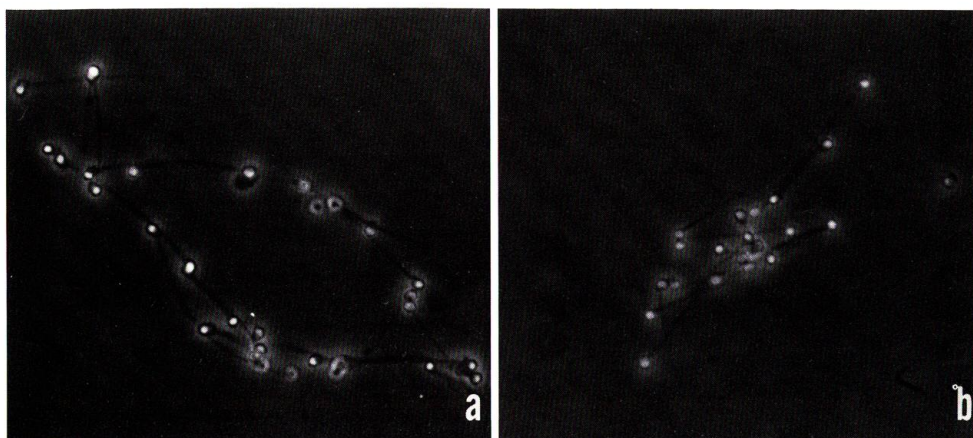


Fig. 2. Agglutination patterns of allogeneic (a) and autologous spermatozoa (b) following incubation with sera from mature male tilapia treated with ATH+FCA. Phase-contrast images.  $\times 410$ .

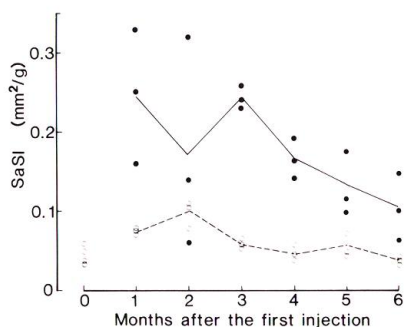


Fig. 3. Changes in spleen area-somatic index (SaSI) of maturing male tilapia treated with ATH+FAC (●) or FCA alone (○) in experiment 1. Lines in the figure are connections of mean values.

### Histology

In experiments 1 and 2, fish of both the control and immunized groups began to mature from 3 months after the first injection and had ripe testes by 6 months. Control fish had normal testes except that some whitish globate structures, which resulted from intraperitoneal injections of FCA, appeared on the surface of the testes. On the other hand, immunized fish showed various pathological changes in their testes only. The fish killed at 1 month of experiment revealed no notable response to injections of ATH+FCA or AS+FCA in their testes, though distended blood vessels, especially those found in interspaces between the efferent ducts, were observed to contain many lymphocytes. In the fish killed at 2 months of experiment, monocytes and lymphocytes had begun to infiltrate locally into testicular interstitial tissue and further invaded into the efferent ducts and seminal lobules of affected testes.

Beginning from 3 months, various changes, including phagocytosis of spermatozoa and formation of granulomatous structures, were observed in testes of immun-

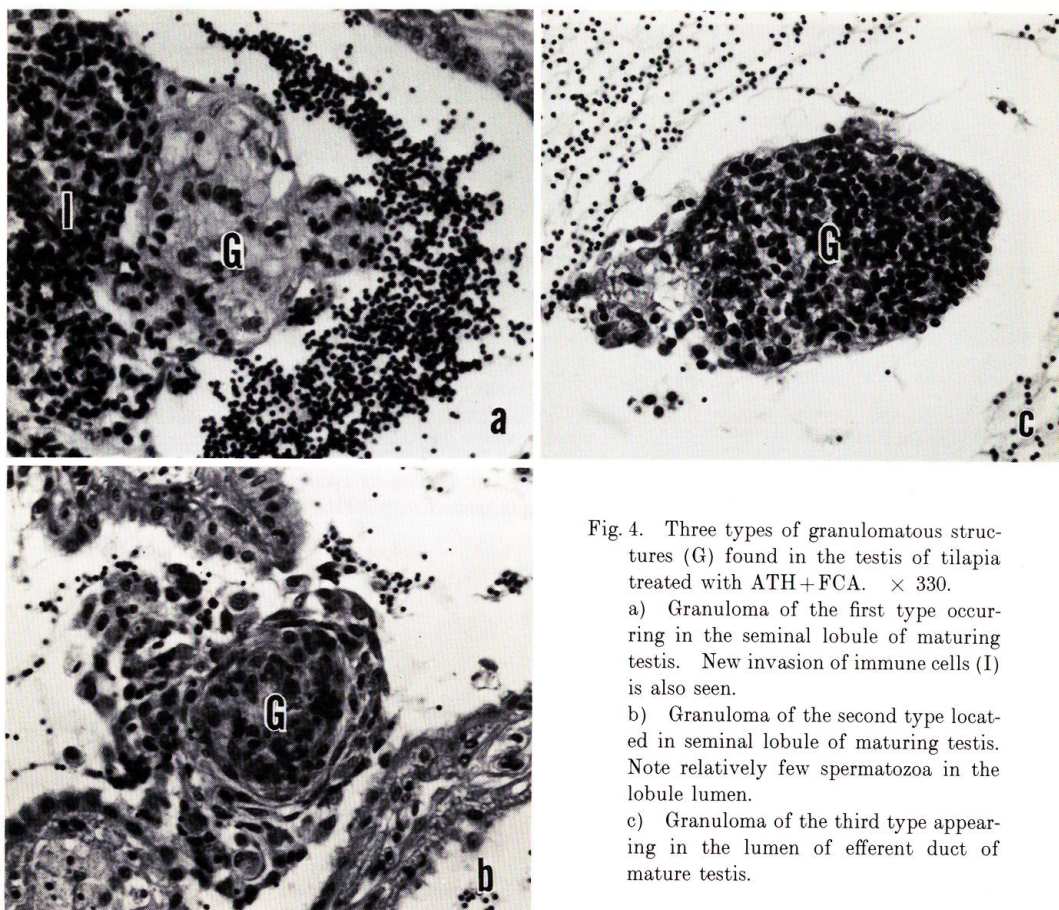


Fig. 4. Three types of granulomatous structures (G) found in the testis of tilapia treated with ATH+FCA.  $\times 330$ .

a) Granuloma of the first type occurring in the seminal lobule of maturing testis. New invasion of immune cells (I) is also seen.

b) Granuloma of the second type located in seminal lobule of maturing testis. Note relatively few spermatozoa in the lobule lumen.

c) Granuloma of the third type appearing in the lumen of efferent duct of mature testis.

ized fish. The granulomatous structures could be divided into three types according to their cellular composition. The structure of the first type, which made its appearance always in lumina of seminal lobules near the exit to efferent ducts, consisted exclusively of giant cells deriving from monocytes (Fig. 4a). Some of the constituent cells characteristically showed vacuolar aspects in the cytoplasm. The structure of the second type was observed to occur in efferent ducts with a few spermatozoa and were composed of monocytes aggregated in concentric layers encircling a core of eosinophilic cells (Fig. 4b). That of the third type was present in efferent ducts of ripe testes only and consisted of a mass of cells covered by a thin fibrous layer (Fig. 4c). The cells were quite similar in aspects to lymphocytes which had formerly invaded into the duct lumen. The invasion of monocytes and lymphocytes into efferent ducts and seminal lobules had ceased 6 months after immunization, despite the fact that infiltration of these cells into testicular interstitium still continued, and no remarkable pathological changes were detectable in testes of some fish sampled at that time.

### Discussion

Results of the present study, together with those reported previously (Lou and Takahashi, 1987), indicate that the immunization of maturing tilapia either with allogeneic testis homogenate or with allogeneic spermatozoa capacitates the serum of the treated fish to agglutinate tilapia spermatozoa, possibly through an elaboration of anti-sperm antibodies. Both the testis homogenate and spermatozoa as antigens induced similar titers of sperm agglutination capacity in the serum, indicating that the spermatozoa are an acutal antigenic source in the present case.

The production of possible autoantibodies against fish spermatozoa was reported for the first time by Secombes et al. (1982) in the rainbow trout, *Salmo gairdneri*, immunized with mature testis material. Secombes et al. (1982, 1987) have stressed that tangle agglutination of spermatozoa is the general pattern caused by antisera from autoimmunized trout, and have suggested that the antigens responsible for such antibodies are present over the whole surface of trout spermatozoa. In the present study, however, agglutination of allogeneic spermatozoa of the tilapia exactly showed the tangle pattern, whereas that of autologous spermatozoa was mostly of the head-to-head pattern. Recent researches have shown a differential distribution of sperm surface antigens in fishes. Jonas-Davies et al. (1983) ultrastructurally and cytochemically demonstrated regionally restricted structural differentiation in spermatozoon of the swordtail, *Xiphophorus helleri*. By using monoclonal antibodies against spermatozoa, Parmentier et al. (1984) suggested the distribution of some antigens on the head and mid-piece but not on the tail of spermatozoa of the common carp, *Cyprinus carpio*. Observations by Jaana and Yamamoto (1984) of agglutination reaction of spermatozoa to some agglutinins in the chum salmon, *Oncorhynchus keta*, demonstrated a restricted distribution of some antigens on the tail. It seems probable that some autoantigens that are common among individuals of the tilapia may be distributed on the head of spermatozoa, and that those distributed in the tail or over the whole surface of spermatozoa may differ in nature among different individuals of the tilapia.

Sperm agglutination titers of the serum of immunized tilapia were retained at a high level from 1 month to at least 7 months after immunization. Similar results have been obtained in autoimmunized rainbow trout in which high agglutination titers to allogeneic spermatozoa were maintained in the serum lasting for 16 weeks (Secombes et al., 1985b). On the other hand, in *Tilapia mossambica* (= *Oreochromis mossambicus*), circulating antibody titers to sheep red blood cells and bovine serum albumin declined rapidly following a peak and became undetectable about 1 month after immunization (Sailendri and Muthukkaruppan, 1974). A chronic presence of circulating antibodies seems to characterize the autoimmunity to testis in fishes. Enlargement of the spleen in immunized fish, as indicated by increased spleen area-somatic index, may be suggestive of the phenomenon. The introduction of allogeneic testis material into fishes may likely cause a chronic leakage of autoantigens from the testis suffered by autoimmune lesions.

An important aspect of initial cellular immune responses to testis material in the tilapia was represented by invasion of monocytes and lymphocytes into seminal lobules of affected testes. The response began to occur by 2 months after immunization, appearing later than the humoral immune response in which sperm agglutina-

tion titers of the serum were at their highest by 1 month after immunization. This suggests the possibility that the induced antibodies may be a necessary factor for immune cells to be activated to break the blood-testis barrier. In fact, the blood-testis barrier of the Nile tilapia, which was ultrastructurally established later than the middle chromatin condensation stage of spermiogenesis, was found to be damaged in the fish immunized to testis material (Lou and Takahashi, unpublished). Using immunofluorescence techniques, Secombes et al. (1985a) could detect trout immunoglobulin in seminal lobules of autoimmunized trout.

Tissue lesions caused in the testis by the present autoimmunization treatment appear to be much weaker than those observed by Secombes et al. (1985a) in the rainbow trout. However, the lesion eventually led to the formation of granulomatous structures in the efferent duct and seminal lobule, as was the case for autoimmune changes of the testis of the rainbow trout (Secombes et al., 1985b). Among the three types of granulomatous structures observed in the tilapia, the former two were regarded as giant cell granulomas composed of the cells of monocyte line which had completed their phagocytotic activities, while the third type appeared to be merely an aggregation of lymphocytes which had been confined in the lumen of seminal lobules. The formation of granulomatous structures as well as other autoimmune lesions did not impair normal spermatogenesis in the testis of immunized tilapia until at least 6 months after immunization. The pathological changes observed in the testis of maturing male tilapia tended to be alleviated as the testis grew to maturity, in spite of high sperm agglutination titers retained in their sera. When mature male tilapia were subjected to immunization, sperm agglutination titers of their sera seemed to decrease at relatively a rapid rate after reaching the peak similar to that found in immunized maturing males. Some structural differentiation in the testis may likely occur with maturation in order to prevent extensive invasion of immune cells into seminal lobules and associated ducts. Thus it seems difficult to completely sterilize maturing and mature male tilapia by the method for inducing autoimmune responses to testis material tested in the present study.

As only late spermatids and spermatozoa of the rainbow trout (Secombes et al., 1985a) and exclusively spermatozoa of the Nile tilapia (Lou and Takahashi, 1987) are attacked by immune cells during autoimmune responses to testis, it is reasonable to consider that some sperm-specific antigens appear on the surface of spermatozoa at some late phase of spermiogenesis. Such antigens have been investigated in the common carp by using monoclonal antibodies (Parmentier et al., 1984). In mammals, some sperm-specific antigens are considered as important factors in fertilization (Tung, 1983). It is interesting to know whether the spermatozoa coated by autoantibodies are able to normally fertilize eggs, since sperm agglutination titers in the serum of immunized tilapia remained high for several months in the present study.

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