Initial Phase of an Inflammatory Reaction in Testis Caused by Experimental Testicular Autoimmunity in the Nile Tilapia, Oreochromis niloticus

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Initial Phase of an Inflammatory Reaction in Testis Caused by Experimental Testicular Autoimmunity in the Nile Tilapia, *Oreochromis niloticus*

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ABSTRACT—In order to study the pathogenesis of the testicular autoimmunity induced by the immunization with allogeneic testis homogenate (ATH) mixed with Freund's complete adjuvant (FCA) in the Nile tilapia *Oreochromis niloticus*, the initiation of inflammatory reactions in the testis was ultrastructurally investigated and seminal plasma was analyzed for presence of autoantigens. In the seminal plasma, mainly 120 kD and 80 kD autoantigens were identifiable by Western blotting with tilapia antisperm autoantibody. Eight weeks after injection with FCA, globate structures, possibly originating from FCA, were large and distended the interstitium where the inflammatory reactions were more frequently recognizable. The globate structures may be one of causes to break the blood-testis barrier, and the inflammatory reaction may be due to the leakage of soluble autoantigens from the lumen. In the interstitium, several kinds of immunocompetent cells formed masses which were mainly composed of lymphocytes, macrophages, eosinophils and plasma cells. We suggest that the inflammatory reactions in fish caused by the immunization with ATH+FCA are initiated by sensitization of the immunocompetent cells with testicular autoantigens, the 120 kD and 80 kD autoantigens in the seminal plasma are two of them, leaking from the lumen because of the provable effect of FCA. These initial reactions were then amplified by cellular and humoral immunity.

INTRODUCTION

Several testicular antigens are known to be immunogenic to the autologus host, and testicular autoimmunity can be experimentally induced by introducing these antigens. (e.g., vasectomy, or immunization with testis homogenate or spermatozoa in Freund's complete adjuvant). The models of testis specific autoimmune disease in mammals have been reviewed in detail by Tung and Menge [22]. Several light- and electron-microscopic studies have been performed with the aim of clarifying the pathogenesis of testicular lesions. Studies on the inflammatory response in the interestitium following vasectomy suggest that an inflammatory reaction is evoked by soluble sperm antigens or constituents of seminal plasma that leaked from the lumen of sperm ducts following distension of ducts with numerous sperm and debris, or by the rupture of the duct system with formation of spermatic granuloma(s) [4, 5]. On the other hand, in inflammatory reactions induced by the injections with testis homogenate, or spermatozoa in adjuvant, the testicular autoantigens are thought to access the immune system in the interstitium following breakdown of the blood-testis barrier by components in Freund's complete adjuvant [22].

As in several mammalian species, testicular autoimmunity can be experimentally induced in some teleosts by immunization with testis homogenate in Freund's complete adjuvant, and prominent inflammatory reactions, such as macrophages phagocytozing spermatozoa, are identifiable in the testis [9–11, 18, 19]. Lou and Takahashi [12] also showed through a tracer study that the blood-testis barrier was broken down during the testicular autoimmune response.

However, in teleosts and other lower vertebrates, no studies have been conducted on the occurrence of an inflammatory reaction in the testis during induced autoimmunity. In the present study, we investigated the pathogenesis of an inflammatory reaction induced by immunization with allogeneic testis homogenate in Freund's complete adjuvant. We attempted to detect autoantigens in the seminal plasma using Western blot analysis by antisperm autoantibody in the Nile tilapia, *Oreochromis niloticus*. In addition, the testis of animals 4 and 8 weeks after the immunization were analyzed ultrastructurally, with particular attention to the characteristics of the responding cells and their interaction in the interstitium.

MATERIALS AND METHODS

Animal

Maturing and mature male Nile tilapia (150–400 g in body weight) *Oreochromis niloticus* used in the present study were maintained in indoor concrete ponds in the campus of the Faculty of Fisheries, Hokkaido University, at 25–30°C under natural light conditions, and fed on a commercial diet (Nippai Fish Food Co.) two or three times a day.

Induction of autoimmunity to testis homogenate

The induction of autoimmune responses in the testis was carried out according to the method described by Lou and Takahashi [11]. Mature testis from freshly killed fish were homogenized at 4°C in a

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glass homogenizer with 0.7% saline to form a 50% solution of testis homogenate. The allogeneic testis homogenate (ATH) thus obtained was emulsified in an equal volume of Freund's complete adjuvant (FCA; nacalai tesque, Kyoto, Japan). Ten fish were immunized with intraperitoneal injections of ATH+FCA, and another ten fish were injected with saline+FCA, at a dose of 2.5 $\mu l/g$ body weight. Nine control male fish received saline injection at the same dose. Injections were given once a week for 3 weeks. Four and 8 weeks after the first injection, 5 fish from each group injected with ATH+FCA, or saline+FCA were killed, and 3 fish injected with saline were killed 0, 4 and 8 weeks after the first injection. Testes were processed for histological observation.

Histology

A median portion of each testis was cut into small fragments which were fixed with a mixture of 1% glutaraldehyde- 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C for 12–24 hr. After washing with the same buffer containing 10% sucrose overnight, the pieces were postfixed in 1% osmium tetroxide in the same buffer for 2 hr at 4°C. The specimens were then dehydrated through a graded acetone series and embedded in Epon. Ultrathin sections (silver to grey) were cut on a Reichert-Jung Ultracut E ultramicrotome, and viewed with a Hitachi H-7000 electron microscope after staining with uranyl acetate and lead citrate. Adjacent semithin sections of the testis were stained with methylene blue for orientation purposes. The classifications of leukocytes followed the criteria proposed in previous studies [1, 3, 20].

Seminal plasma

Milt stripped directly from the mature male fish was centrifuged at 8000 g for 15 min at 4°C. The resultant supernatant was collected as seminal plasma. The seminal plasma was stored at −80°C until electrophoretic analysis.

Electrophoresis

Two-dimensional electrophoresis (2D-PAGE) was performed by the method of O'Farrell [17] using 4 % polyacrylamide gels in the presence of 8 M urea and 2% ampholine encomprising a pH range of 3.5–9.5 for isoelectric focusing, and 7.5–20 % polyacrylamide gradient slab gel for sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were stained with silver (Silver Stain II Kit, Wako). Protein molecular weight markers (low and high MW set) were obtained from Pharmacia (Pharmacia Fine Chemicals, Uppsala, Sweden).

Western blotting

Proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membranes (PVDF; Millipore, Bedford, MA, USA) with a Bio-rad semidry trans-blot SD [21]. The following procedures were done at room temperature. Before immunostaining, each membrane was treated for 30 min with 20 mM Tris-HCl buffer (pH 7.5), 500 mM NaCl (TBS), containing 5% skim milk to block the non-specific protein binding sites. Membranes were then incubated with antisperm autoantibody overnight followed by rabbit anti-tilapia IgM antiserum. The antisperm autoantibody and rabbit anti-tilapia IgM antiserum were prepared by the method described by Lou *et al.* [14]. Finally, membranes were incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Bio-rad, dilution 1 : 1000) for 3 hr. After washing with TBS, membranes reacted with HRP conjugated antibody were incubated with 0.06% 4-chloro-1-naphtol in TBS containing 0.06% H_2O_2 to visualize the peroxidase reaction products.

RESULTS

Autoantigen detection

Electrophoretic patterns and Western blot analysis by antisperm autoantibody of the seminal plasma are shown in Figure 1. The 2D-PAGE analysis of seminal plasma revealed 120 kD polypeptide, which migrated in a pH range greater than 9.0, and several other polypeptides of 80, 67, 26 and 19 kD. The last four polypeptides migrated in acidic pH ranges of 4.5–5.0, less than 5.0, 6.0–7.0, and 5.5–6.5, respectively. The 120 kD and 80 kD polypeptides showed positive reactions with antisperm autoantibody and the 120 kD polypeptide reacted strongly. Another 120 kD polypeptide which migrated in the pH range 6.0–6.5 also reacted with the autoantibody, but the polypeptide could not be detected on the gel with silver staining.

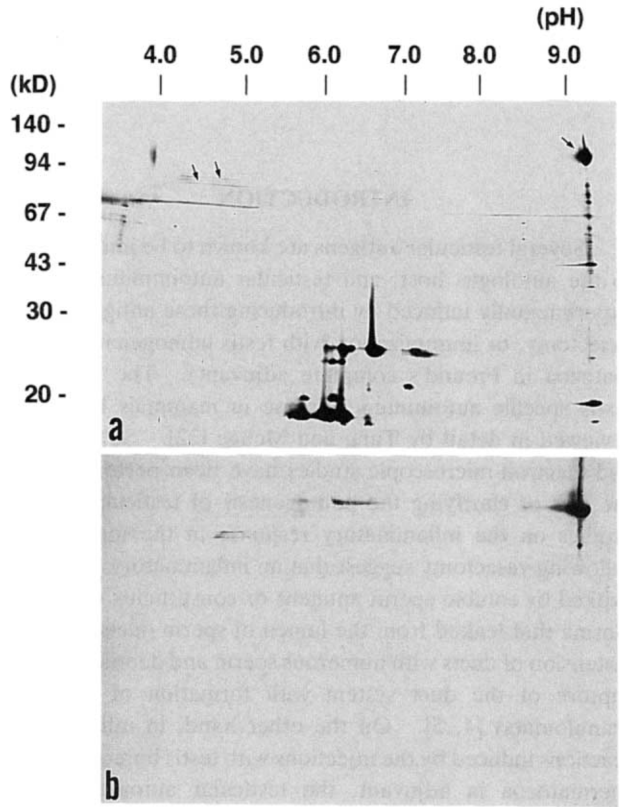

Fig. 1. Two-dimensional gel analysis with Coomassie Brilliant Blue staining (a) and Western blot analysis by antisperm autoantibody (b) of the seminal plasma of the Nile tilapia under reducing conditions. Positions of molecular weight markers and isoelectric markers are indicated on the left and the upper side of the figure, respectively. Arrows show the proteins which reacted positively with the autoantibody.

Histopathological changes

The histology of the normal tilapia testis has been described in detail [13]. To summarize, the testis is composed of interstitium and seminal lobules. These two compartments are separated by a basement membrane. Leydig cells and myoid cells are typical of the interstitium, whereas the seminal lobule contains both Sertoli cells which form cysts, and germ cells. Germ cells in the seminal lobules are packed into the cysts which are arranged in a single layer on the wall of seminal lobules.

In fish killed 4 weeks after the first injection with ATH+

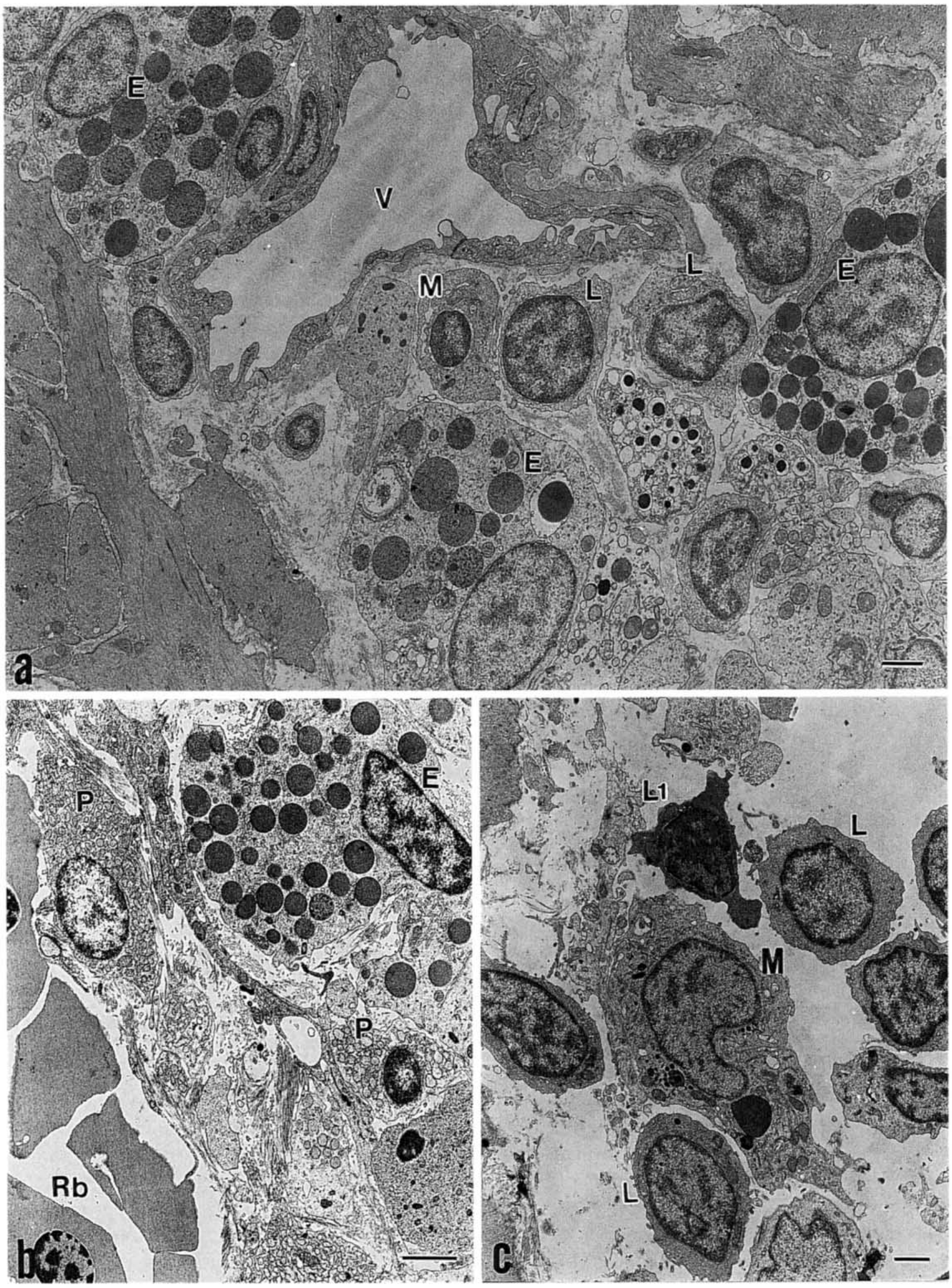

FIG. 2. Interstitium of the testis 8 weeks after injection with ATH+FCA. Some of the interstitial areas are occupied by a variety of cell types (Fig. 2a), including macrophages (M), lymphocytes (L), and eosinophils (E). Plasma cells (P) are present in Fig. 2b and another type of lymphocyte (L1) is shown in Fig. 2c. Around the lymphocyte, degeneration of testicular somatic cells is prominent. V, blood vessel Rb, red blood cell. Bars, 1 μm.

FCA, few distinct pathological changes were noticeable in the testis. Leukocytes, mainly lymphocytes and monocytes, slightly increased in number in the interstitial tissues. The characteristics of these leukocyte have been described previously [15].

In the testes of fish killed 8 weeks after injection with ATH+FCA, some areas of the interstitial spaces were expanded. Ultrastructurally, the interstitial region was characterized by irregularly arranged myoid cells and randomly scattered collagen fibrils. The structure of many collagen fibers was obscured. In addition, this area was occupied by many leukocytes of a variety types, including lymphocytes, macrophages, eosinophils and plasma cells (Fig. 2). The lymphocytes were often in the vicinity of macrophages, and some of the lymphocytes attached with the macrophages (Fig. 3). Plasma cells, distinctive because of their well-developed rough endoplasmic reticulum, Golgi apparatus and roundish nuclei were also evident in the interstitium (Fig. 4a, b). Cisternae of rough endoplasmic reticulum were quite distended. The plasma cells were usually spherical in shape, about 8 μm in diameter, but sometimes they had an irregular contour and had cytoplasmic processes. The eosinophils were spherical in shape, about 10 μm in diameter, and displayed heterochromatin, moderate numbers of mitochondria, some vacuoles and many homogeneously electron-dense granules about 0.5 μm in diameter in their cytoplasm (Fig. 4c). Several of these granules were composed of many smaller electron-dense aggregates. The endoplasmic reticulum was located particularly around large granules about 3 μm in diameter. In a few instances, another kind of lymphocyte was also recognized, characterized by a diameter of 3–4 μm heterochromatic nuclei, electron-dense cytoplasm and small vacuoles in their cytoplasm (Fig. 4d). The lymphocytes occasionally developed their cytoplasmic processes and attached to somatic cells such as myoid cells, Sertoli cells and epithelium of seminal ducts. Degeneration of testicular somatic cells was prominent around the regions where the lymphocytes were detected.

Globate structures, which seemed to be unabsorbed liquid paraffin component of FCA, were surrounded by thin layers of connective tissues and adhered to the surface of the testis in fish killed 4 weeks after injection (Fig. 5a). In fish killed 8 weeks after injection, these structures were distributed in the interstitium around efferent ducts, and were larger (Fig. 5b). Around this area, the histopathological reactions tended to be prominent. Globate structures possibly originating from FCA were also observed in the testes of fish injected with saline+FCA. Eight weeks after injection, these structures grew larger in the interstitium. However, almost no immunocompetent cells were recognized except for a small number of eosinophils.

Except for the areas of interstitial reactions containing adjacent basement membrane and Sertoli cells, testicular tissues of fish injected with ATH+FCA or saline+FCA showed the same appearance as that of testis of fish injected with saline.

DISCUSSION

The present electrophoretic and Western blot analysis by the antisperm autoantibody revealed several kinds of autoantigens in the seminal plasma. The 120 kD polypeptide in

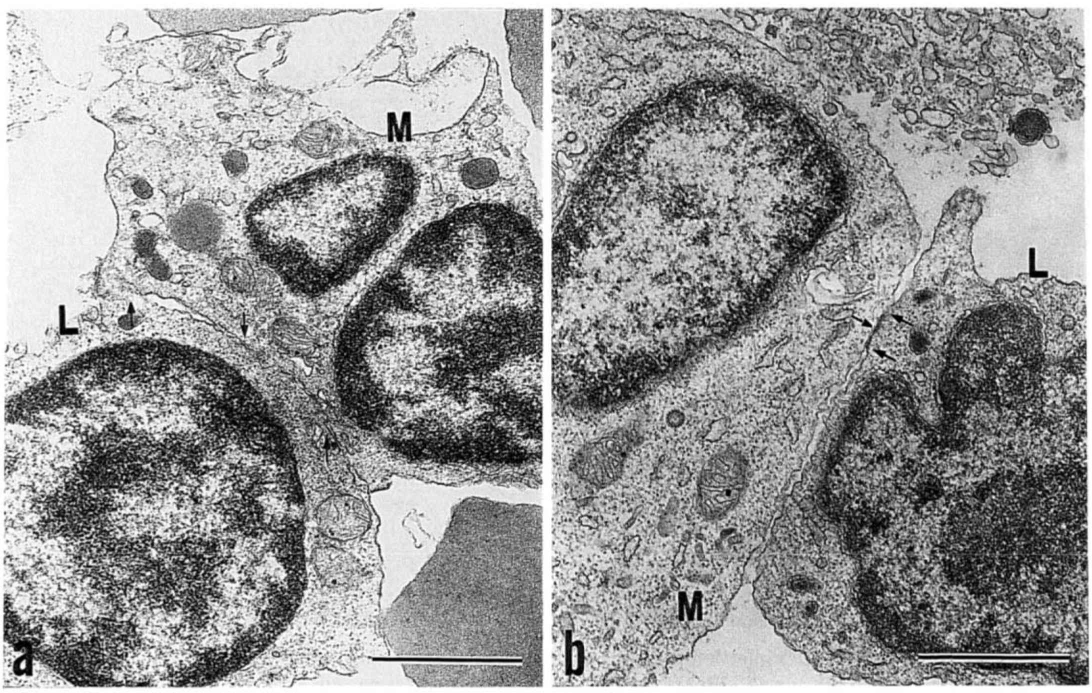

FIG. 3. Interstitial regions in fish 8 weeks after injection with ATH+FCA. Macrophages (M) are closely interdigitated with lymphocytes (L). Arrows show the regions of interdigitation. Bars, 1 μm.

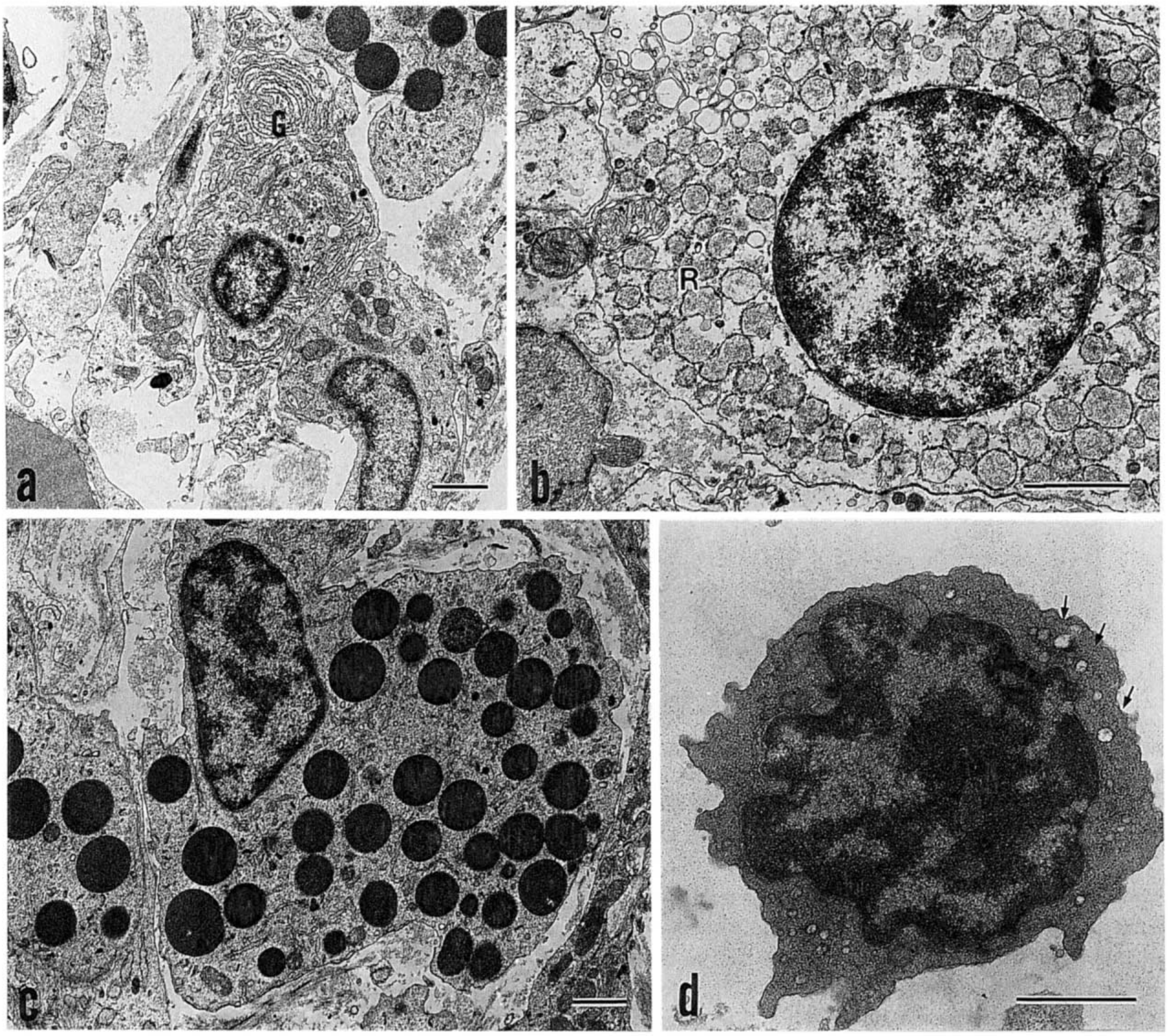

FIG. 4. Enlarged views of several kinds of immunocompetent cells detected in the interstitium of fish 8 weeks after injection with ATH+FCA. Plasma cells have well-developed Golgi apparatus (G, Fig. 4a) and rough endoplasmic reticulum (R, Fig. 4b) which contain electron-dense material in their cisternae. An eosinophil has many homogenously electron-dense granules in its cytoplasm (Fig. 4c). Another type of lymphocyte has several small vacuoles (arrows in Fig. 4 d) in its electron-dense cytoplasm and sometime developed cytoplasmic projections. Bars, 1 μm.

the pH range of more than 9.0 and the 80 kD polypeptide in the pH range 4.5–5.0 were identified as autoantigens, with the 120 kD autoantigen showing strong immunogenicity. Another 120 kD polypeptide in the pH range 6.0–6.5 was present in very small amounts. The isoelectric point of this polypeptide was possibly changed by the treatment for electrophoresis such as urea. In spite of this, it was originally the main 120 kD polypeptide in the pH range more than 9.0. To our knowledge, there is no direct evidence from mammalian studies that seminal plasma contains autoantigens. The 120 kD and the 80 kD autoantigens in the seminal plasma may be the immunogens which induce the inflammatory reaction. However, it is not appear that these autoantigens can induce the inflammatory reaction, because the ATH may contain many antigens which have ability to cause inflammatory reaction. It is future study to analyze the immunization of the 120 kD and the 80 kD autoantigens can induce the inflammatory reaction.

The leakage of soluble antigens into the interstitium is thought to be necessary for induction of the inflammatory reaction, and is caused by the distension of duct system with numerous sperm and debris in the case of vasectomy [4, 5]. The globate structures observed in the present study, which are composed of connective tissue surrounding unabsorbed liquid paraffin component of FCA, may be one of factors to induce the leakage of the seminal plasma to the interstitium. The structures appeared in the interstitium around the main sperm duct 8 weeks after injection with FCA. In some of these regions, the interstitium was extensively distended by the structures. It is plausible that the globate structures cause the breakdown of blood-testis barrier, and then leakage of soluble autoantigens to the interstitial tissue follows. The

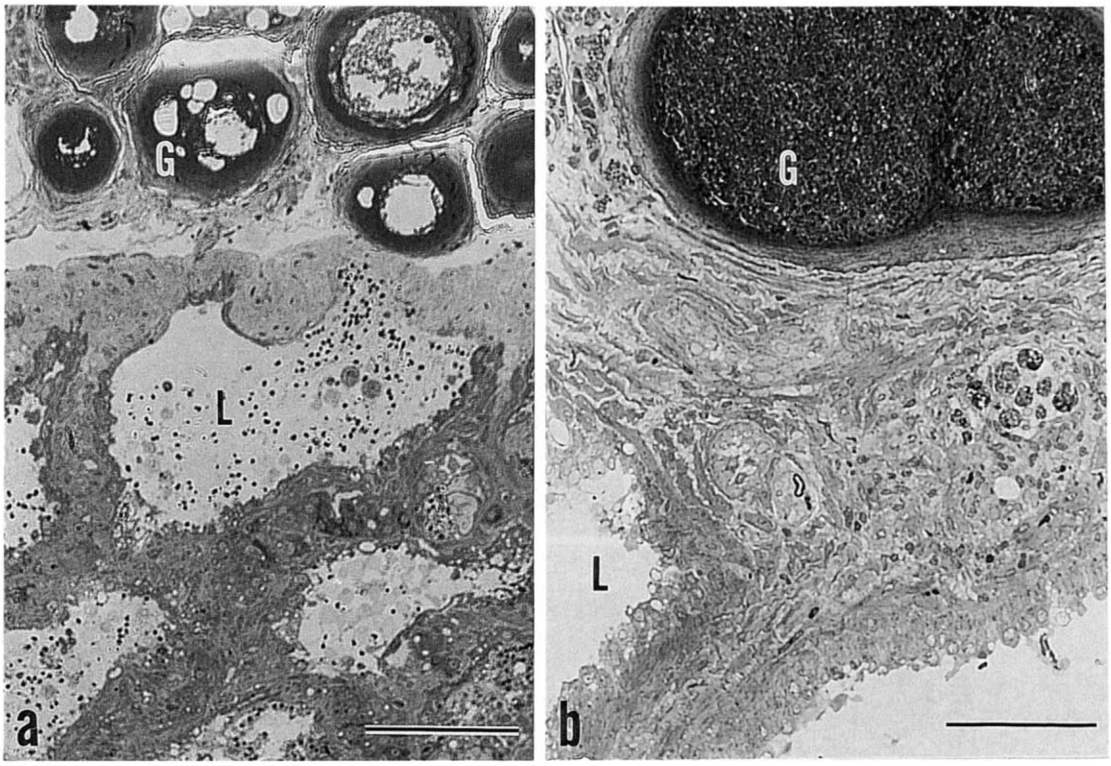

Fig. 5. Globate structures (G) which were composed of connective tissues surrounding some chemical component of FCA. In the fish 4 weeks after injection with saline+FCA and ATH+FCA, the structures were observed on the testis capsule (Fig. 5a), but after 8 weeks past injection, the structures developed and distended the interstitium (Fig. 5b). L, seminal lobule lumen. Bars, 50 μm.

observation that the histopathological reactions tended to be prominent around this area also supports the idea that the globate structures are one of the factors involved in breakdown of the blood-testis barrier. In the guinea pig, injection with FCA+saline caused a breakdown of blood-testis barrier suggested that the barrier of the testis was weakened by some chemical constituents of FCA [23]. Further studies are needed to clarify the mechanisms of breakdown of the blood-testis barrier during the autoimmune response induced by the immunization using FCA.

Recently we showed that the 120 kD autoantigen was localized in the cytoplasm of Sertoli cells and epithelial cells of efferent ducts, and we suggested that it is synthesized and secreted by Sertoli cells and epithelial cells of seminal ducts [16]. As in the rat testis [6], the basement membrane of the Nile tilapia testis also appears to act as an ion-selective filter, because it allows cationic proteins to pass through, but not anionic proteins at their isoelectric points of 8.5 and 4.6, respectively [12]. According to these authors, the 120 kD autoantigen is cationic and is thought to be able to pass through the basement membrane and possibly exists normally outside the blood-testis barrier. There is no direct evidence in mammals and teleosts that the testis-specific autoantigens are completely sequestered from the immune system. Further, there is evidence for serum autoantibodies reacting with testicular germ cells located outside the blood-testis barrier in the mouse [24]. Tung and Menge [22] provided one hypothesis of immunological unresponsiveness to testis-specific autoantigens. Relating this hypothesis to the Nile tilapia, the 120 kD autoantigen may normally exist outside the blood-testis barrier in testis, but immunological tolerance to the autoantigen normally prevents the induction of testicular autoimmunity. However, autoimmune disease is caused when the tolerance state may be terminated by injection with the appropriate adjuvants. In mouse, Sertoli cells secrete molecules capable of inhibiting proliferation of B or T lymphocytes *in vitro* [2]. It is necessary to further define the exact localization of the 120 kD autoantigen, the roles of Sertoli cells in immunosuppression and the effects of FCA on immunological tolerance in the Nile tilapia.

The most prominent change in the interstitium of testis after the injection with ATH+FCA was aggregations of large numbers of cells with ultrastructural characteristics of macrophages, lymphocytes, eosinophils and plasma cells. The characteristics of the interstitial lesions are similar to those observed in the rat epididymal interstitium after vasectomy, which indicated chronic inflammatory reactions [5]. The ultrastructural characteristics of the immunocompetent cells suggests their possible roles in the autoimmune reactions.

Macrophages sometimes made contact with lymphocytes. Previous studies on cell-mediated immunity in teleosts suggested the presence of products of a major histocompatibility system on cells [7]. In addition, in testicular autoimmunity of some teleost fish, immune reactions were apparently specific to spermatozoa which expressed the autoantigens on their surface [10, 18]. These results suggested that the macrophages provided an opportunity for presentation of the autoantigens to the lymphocytes. The images of the macrophages also suggested that the autoimmune inflammatory reactions were initiated in the interstitium of the testis of immunized fish.

Many eosinophils containing many granules of varying shapes were also recognized in the areas of interstitial reactions, and they appeared to be actively phagocytozing some types of molecules. However, this type of eosinophils has lower capacity for phagocytosis than macrophages and neutrophils in some teleost fish [3, 20]. It is necessary to further investigate the functions of the eosinophils observed in the present study. In murine experimental allergic orchitis induced by immunization with homologous testicular tissue homogenate emulsified in FCA, large numbers of eosinophils were detected inside and outside the seminiferous tubules, although their roles are as yet unknown [8].

One of the characteristic images detected in the interstitium of immunized fish was the plasma cell, which was not recognizable in the testis of fish injected with FCA+saline or saline. Ultrastructural features of the plasma cells suggested active synthesis and secretion of antibodies. These observations are an accurate reflection of previous work showing that the sperm agglutination titre of fish injected with ATH+FCA, which represented the ratio of the amount of specific antibodies to sperm antigens in the serum, was significantly higher than those of fish injected with FCA only [15]. Also, the inflammatory reaction could not be induced by the injection with FCA only. These results strongly suggests that humoral immunity is a prerequisite for the occurence of inflammatory reactions.

The other characteristic image was another kind of lymphocyte which had electron-dense cytoplasm and several small vacuoles. Although the lymphocytes may play an important role in the lysis of those somatic cells and the increased permeability of the testicular somatic cells to macrophages, much still remains to be done before identifing the function of this kind of lymphocyte.

The present study suggests that much of the pathogenesis of testicular autoimmunity in a lower vertebrate, the Nile tilapia, is similar to that proposed in mammalian studies [5, 22].

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