Virchows Arch [Pathol Anat] (1981) 392:147–158

Virchows Archiv A

Experimental Allergic Orchitis in Mice

Histopathological and Immunological Studies

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Summary. Experimental allergic orchitis was induced in (C57BL/6J × A/J)F_1 mice by two injections of syngeneic testicular homogenate emulsified with adjuvants immediately followed by intravenous injection of pertussis vaccine, at a 2 week interval.

Histologically, in the initial stage there was occasional focal degeneration and desquamation of both spermatogonia and Sertoli cells within limited parts of the seminiferous tubules, in the peripheral region of the testis. No inflammatory change was present. In some cases, however, inflammatory reaction in the rete testis and focal lymphocytic infiltration in the interstitium were also observed. Subsequently, marked infiltration of lymphocytes, monocytes, and polymorphs were found not only in the testes, but also in rete testis and epididymis. In later stages the inflammatory reaction gradually subsided, but the testes became atrophic due to progression of spermatogenic arrest. Many tubules were lined only with monolayers of Sertoli cells, surrounded by hyperplastic Leydig cells in the interstitium. At 5 months after the 2nd immunization, there was still variable depression of spermatogenesis and hyperplasia of Leydig cells with scattered fibrous scars in the seminiferous tubules, although good regeneration of germ cells appeared in some tubules.

Immunological studies revealed that lymphocytes obtained from mice bearing developed orchitis showed a significantly enhanced response in the mixed culture with syngeneic testicular cells, and suggest that cellular immunity plays an important role in the induction of experimental allergic orchitis in mice.

Key words: Orchitis – Autoimmune diseases – Spermatogenesis – Sertoli cells – Leydig cells

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0340-1227/81/0392/0147/02.40

Introduction

Freund et al. (1953) succeeded in producing aspermatogenesis in the guinea pig by injection of testicular homogenate emulsified with Freund's complete adjuvant. Since then, there have been many reports on experimental allergic orchitis in guinea pig from either histopathological or immunological view points, because of the ease with which the lesion is induced in this animal. Though inbred mice would be an undoubtedly better choice for a model animal, it has been rather difficult to produce experimental allergic orchitis in mice. There have been only a few reports of murine experimental allergic orchitis (Pokorná et al. 1963; Boehme 1965; Hargis et al. 1968; Bernard et al. 1978), and a systemic histological observation has not yet been documented in mice.

We produced experimental allergic orchitis in inbred mice, according to the method of Bernard et al. (1978), and observed systematically the histopathological changes in the testes and a correlation between testicular lesion and cellular immunity. The mechanism and site of the initial immune reaction against germ cell antigen in the testes of the immunized animals is also discussed.

Materials and Methods

Mice. Male (C57BL/6J × A/J)F_1 mice (hereafter referred to as BAF_1), were used ranging in age from 10 to 22 weeks. The female parents, C57BL/6J mice, were bred in the colonies at the Institute of Medical Science, University of Tokyo. The male parents, A/J mice, were bred in Jackson Laboratory. BAF_1 mice were bred in the laboratory of Department of Pathology, Tokyo Medical and Dental University. The male mice examined were sixty-six in total.

Antigen. The syngeneic testes were decapsulated, minced and homogenized with an equal amount (v/w) of saline in a homogenizer and used as testicular antigen.

Immunization Procedure. The procedure was mostly according to the method of Bernard et al. (1978). The testicular homogenate was emulisified with an equal volume of FCA containing 0.5 mg of mycobacterium butyricum (Iatron Laboratories, Tokyo), and 4 mg/ml of mycobacterium tuberculosis (Institute of Medical Science, University of Tokyo). The emulsion was injected subcutaneously into the four foot pads of mice, followed by intravenous injection of pertussis vaccine (BPV: Bordetella pertussis vaccine, Chiba Kessei Laboratories, Chiba) containing 1×10^{10} organisms. Controls were injected with saline emulsified with same adjuvants. After two weeks interval, the same antigenic emulsion was injected subcutaneously into four foot pads and back skin, immediately followed by intraperitoneal injection of BPV (5×10^9). At 5 months after the 2nd immunization, testicular homogenate emulsified with adjuvants was injected in the same manner as before, followed by intravenous injection of pertussis vaccine.

Histopathology. At 2 weeks and 6 weeks after the 1st immunization, 2, 4, 8 weeks and 5 months after the 2nd immunization, and 2 weeks after the 3rd immunization, an appropriate number of mice (from 3 to 5 for each group) were killed and used for histopathological and immunological studies.

The testicular samples were fixed with Bouin's solution and prepared for histopathological examination. Sections obtained from the testes at 5 months after th 2nd immunization and controls were examined histometrically, in order to estimate areas occupied with Leydig cells.

Mixed Lymphocyte Culture with Testicular Cells. A free suspension of testicular cells was prepared after the method described by Hurtenbach et al. (1980). The testes, freed from capsule, were incubated in 0.1% collagenase (Sigma, St. Louis) dissolved in PBS at 37° C for 12 min. The supernatant, freed from the tubular segments, was collected and designated as interstitial cells, which were composed of 60% free extratubular cells (mainly Leydig cells) and 40% contaminated germ cells.

The tubular segments were then broken up by an incubation in 0.025% trypsin (Difco, Detroit), dissolved in Ca^{++} and Mg^{++} free PBS at 37° C for 15 min, yielding a suspension composed of over 90% germ cells (designated as germ cells). Another cell suspension was made by mixture of equal part of interstitial cells and germ cells (designated as "both" cells).

Lymphocytes (5×10^5) from spleens or lymph nodes were cocultivated with these testicular cells (2×10^5) in 0.2 ml of RPMI 1640 supplemented with 5% FCS and Kanamycin (0.06 mg/ml) for 4 days using microplate (Falcon 3042).

Six hours before harvesting, 0.25 μCi/0.005 ml of ^{3}H-thymidine was added to each well and cells were harvested with a Cell Harvester (model-101 Laboscience, Tokyo).

Results

1. Histology of Experimental Allergic Orchitis in Mice

a) 2 Weeks After the 1st Immunization. At this time there was no remarkable difference in external appearance of the testes between the experimental and control mice. Compared with controls, spermatogenesis was still well preserved in most places (Figs. 1, 2). In the peripheral region of the testes, however, there were occasionally focal areas of degeneration and desquamation of the tubular contents. Such lesions took place in a very limited part of the seminiferous tubules involving adjoining tubules (Fig. 1). It was notable that cell degeneration occurred more intensively in spermatogonia and Sertoli cells which were lying on basal lamina of the tubules than in other mature germ cells. There was no inflammatory cell infiltration in these lesions. In some cases, focal infiltration of lympho-monocytic cells was observed around the seminiferous tubules, although obvious inflammation involving whole tubular wall was not found (Fig. 2). In others there was dilatation of the rete testis containing aggregation mass of sperms and polymorphs, with lympho-monocytic infiltration in the surrounding interstitium, but no change was seen in Leydig cells or epididymis except for slight interstitial edema.

At 6 weeks after the 1st immunization, there was a minimal inflammatory reaction in the interstitium, although a few focal areas of degeneration of germ cells remained in the subcapsular areas.

b) 2 Weeks After the 2nd Immunization. Compared with the size of control testes, the experimental testes became larger or smaller with no consistent finding in their size. There was depressed spermatogenesis, associated with either focal or extensive infiltration of lymphocytes, monocytes and polymorphs (Fig. 3). The most severely involved tubules contained a central mass of necrotic spermatogenic cells and polymorphs (Fig. 3). In other parts without cellular infiltration, the tubules were dilated and lined by thinned layer of germ cells with occasional desquamation and moderate interstitial oedema, but no significant change was observed in Leydig cells at this stage. In almost all cases, the rete testis was dilated and obliterated by numerous necrotic masses of sperms and polymorphs (Fig. 4), associated with an inflammatory reaction. There was also marked infiltration of polymorphs and lymphomonocytes in the interstitium of epididymis. The tubules were sometimes lined by stratified epithelium and contained numerous necrotic masses of sperms and polymorphs (Fig. 5). The change observed in the epididymis was also found in the spermatic cord.

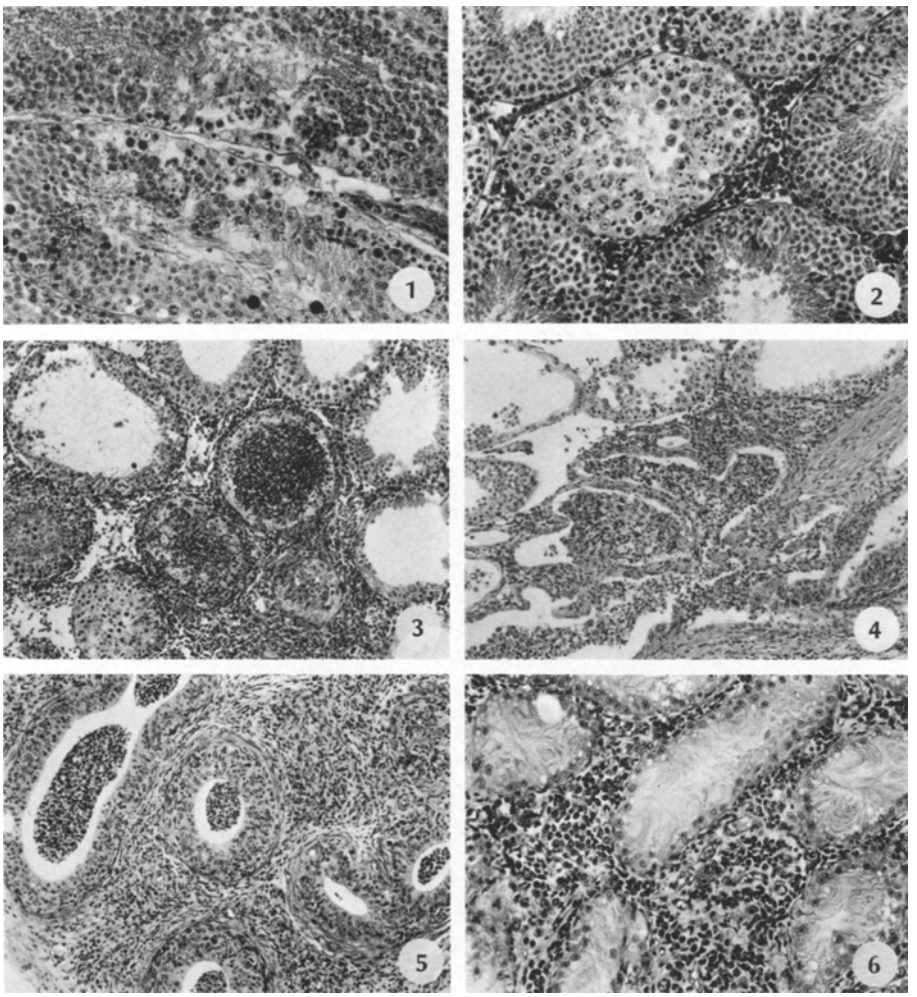

Fig. 1. 2 weeks after the 1st immunization. Focal degeneration and desquamation of the spermatogonia and Sertoli cells in a very limited part of the seminiferous tubules without inflammatory reaction (center of the figure). ×200

Fig. 2. 2 weeks after the 1st immunization. Slight lympho-monocytic infiltration around the seminiferous tubules. Slightly depressed spermatogenesis in the tubule in the center of the figure. ×200

Fig. 3. 2 weeks after the 2nd immunization. Marked aggregation of sperms and polymorphs in the tubules, surrounded by lympho-monocytic infiltration. ×200

Fig. 4. 2 weeks after the 2nd immunization. Marked aggregation of sperms and polymorphs in the rete testis. ×200

Fig. 5. 2 weeks after the 2nd immunization. Marked lympho-monocytic infiltration in the epididymis with aggregation of sperms and polymorphs in the tubules. Stratification of the epithelial cells. ×200

Fig. 6. 4 weeks after the 2nd immunization. Atrophic tubules lined by only Sertoli cells with marked lympho-plasmocytic infiltration in the interstitium. ×200

c) 4 weeks After the 2nd Immunization. The testes became atrophic, about half the size of the controls, and with fibrous thickening of the capsule. Spermatogenesis was markedly and diffusely depressed throughout the testes and no sperm was seen in most places. This change was associated with prominent and extensive lymphoplasmocytic and monocytic infiltration (Fig. 6). The tubules were frequently devoid of germ cells, lined only with Sertoli cells, being surrounded by lymphoplasmocytic infiltration in the interstituum (Fig. 6). In those tubules where germ cells were still preserved, multinucleated giant cells, thought to be made by spermatid cell fusion, were occasionally seen (Fig. 7). There was also frequent vacuolar formation in the tubular contents (Fig. 8). It was not clear whether these vacuoles were inside the Sertoli cell cytoplasm or in intercellular space following germ cell sloughing. The interstitial edema became prominent, and Leyding cells appeared to increase in number. In the epididymis, the acute inflammatory reaction had already declined, but moderate lymphoplasmocytic infiltration still remained. The tubules of the epididymis, lined by single layer of epithelium, were dilated and contained a certain amount of desquamated germ cells and eosinophilic exudates.

d) 8 Weeks After the 2nd Immunization. The testes recovered slightly in size, but were still atrophic compared with controls. Marked depression of spermatogenesis was observed throughout the testes and there were numerous tubules lined by single layer of Sertoli cells with vacuolar formation, probably in the cytoplasm (Fig. 9). Inflammatory reaction had almost ceased, leaving several foci of lymphocytic infiltration. In those tubules where germ cells were present, desquamation was still seen. Moderately hyperplastic Leydig cells, occasionally showing foamy cytoplasm (Fig. 9) filled the wide interstitium. Slight focal fibrosis was occasionally seen in places. At this stage, inflammatory change of the rete testis had subsided completely. In the epididymis, inflammatory reaction became minimal, but dilated tubules still contained a certain number of desquamated germ cells.

e) 5 Months After the 2nd Immunization. The testes were still atrophic, compared with the control. Inflammatory change resulted in several tubular fibrous scars (Fig. 10) and hyperplasia of Leydig cells. The hyperplasia of Leydig cells was confirmed by the histometrical study; i.e., the increase in area occupied by Leydig cells in the testes with orchitis was greater than the expected increase brought about by simple shrinkage of the testes (Table 1). Depressed spermatogenesis was still observed although some of the tubules were lined with regenerated germ cells. The tubules were irregular in size, showing either almost normal appearances or atrophic features. Some of the atrophic tubular interior was made up of Sertoli cells only, showing vacuolar formation. In the epididymis, slight fibrosis was observed around the tubules which contained no sperm.

f) 2 Weeks after the 3rd Immunization. At 2 weeks after the 3rd challenge, histological findings revealed that intensive cellular infiltration and degenerative change in the seminiferous tubules once again appeared in the testes where the inflammation had subsided (Fig. 11), and the changes were more accelerated, compared with those in testes at 2 weeks after the 2nd immunization.

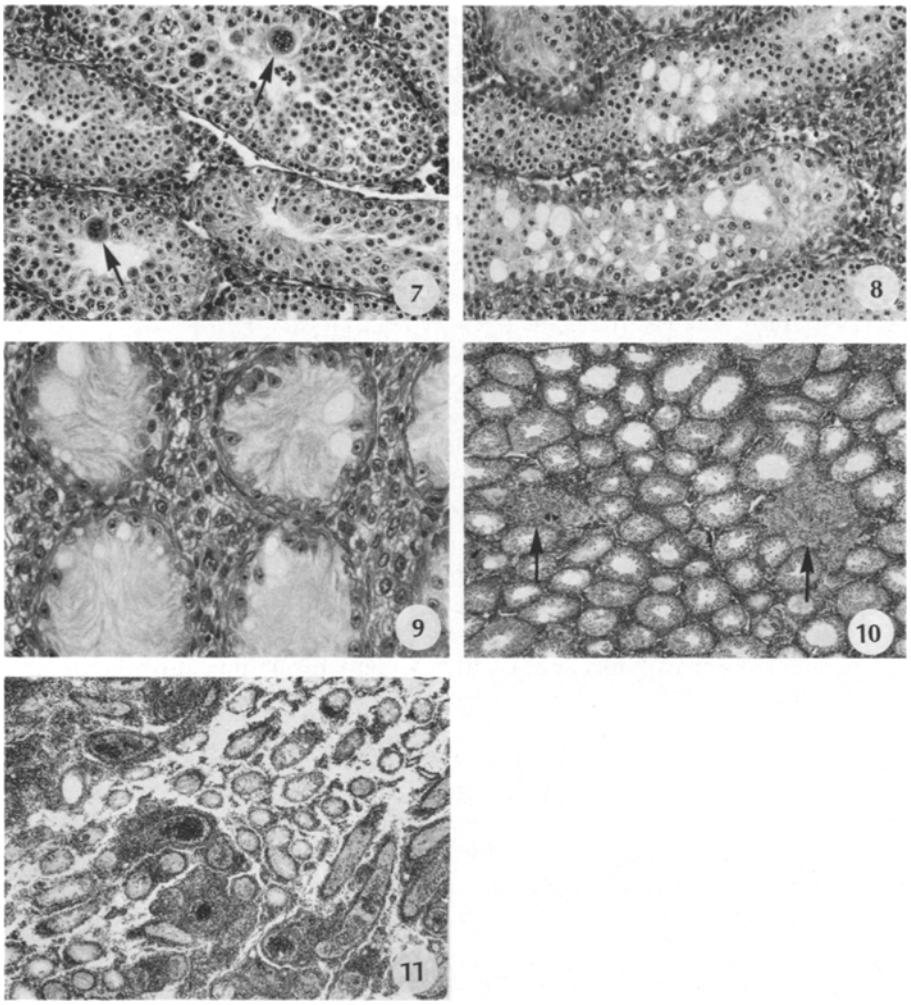

Fig. 7. 4 weeks after the 2nd immunization. Relatively well preserved germ cells, but presence of multinucleated giant cells (arrows) derived from germ cells, and disappearance of sperm. ×200

Fig. 8. 4 weeks after the 2nd immunization. Multiple vacuolar formation in the lumens of the seminiferous tubules, and hyperplasia of Leydig cells. ×200

Fig. 9. 8 weeks after the 2nd immunization. Markedly atrophic tubules lined by only Sertoli cells with intracytoplasmic vacuoles, some of which being in touch with nuclear membrane. Prominent hyperplasia of Leydig cells and little inflammatory reaction. ×400

Fig. 10. 5 months after the 2nd immunization. Two fibrous scars (arrows). Relatively well regenerated germ cells, but remaining or irregular tubular lumens with depressed spermatogenesis. ×20

Fig. 11. 2 weeks after the 3rd immunization. Marked and diffuse infiltration of inflammatory cells with devastation of the seminiferous tubules. ×20

Table 1. Hyperplasia of Leydig cells in the testes with orchitis confirmed by histometrical study

	Number of samples	Area measured at the largest section mean ± 1 S.D. (mm^2)	Area occupied with Leydig cells mean ± 1 S.D. (mm^2)	Ratio of area occupied with Leydig cells in a whole area mean ± 1 S.D. (%)
Control	3	20.4 ± 1.9	1.0 ± 0.2	5.3 ± 1.5
Experiment	3	13.3 ± 1.2	2.3 ± 0.4	17.4 ± 2.0

The area measured at the largest section of the testis with orchitis is about 65% of the control by shrinkage after the cessation of the inflammation. When there is no real increase of Leydig cells in the experimental group, the expected ratio of the area occupied with Leydig cells will be 8.1%. The value is distinctly smaller than that (17.4%) observed in the experimental group, suggesting the presence of real hyperplasia of Leydig cells in the testis with orchitis.

Histometrical measurement was made on the testes at 5 months after the 2nd immunization in both control and experimental groups. Sections stained with hematoxylin eosin were photographed. The areas occupied with Leydig cells were painted on the photographs, cut and weighed. The weight was converted to area (mm^2) by the equation:

$$a(mm^2) = \frac{10^4 \cdot W}{A \cdot M^2}$$

A = weight of photopaper of 10^4 mm^2, M = magnification of the photograph, W = weight of photopaper cut off.

The presence of Leydig cells was always confirmed through microscope during painting procedure

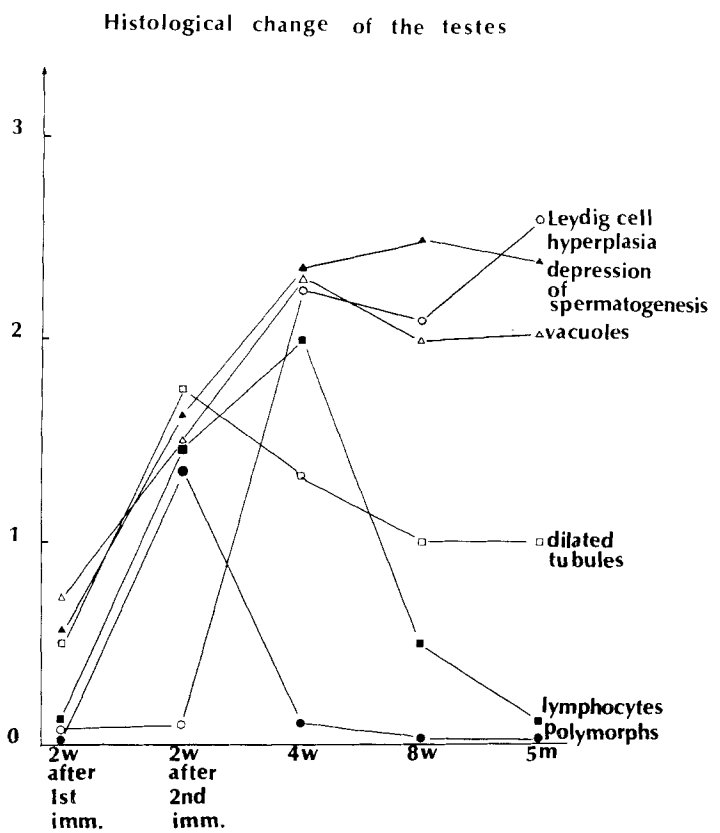

Fig. 12. Chronological observation of the histopathology of the testes, at 2 weeks after the 1st immunization, and 2 weeks, 4 weeks, 8 weeks, and 5 months after the 2nd immunization. Point from 0 to 3 was given to the severity of each change of the testes and calculated mean value of each change obtained from several mice at various stages

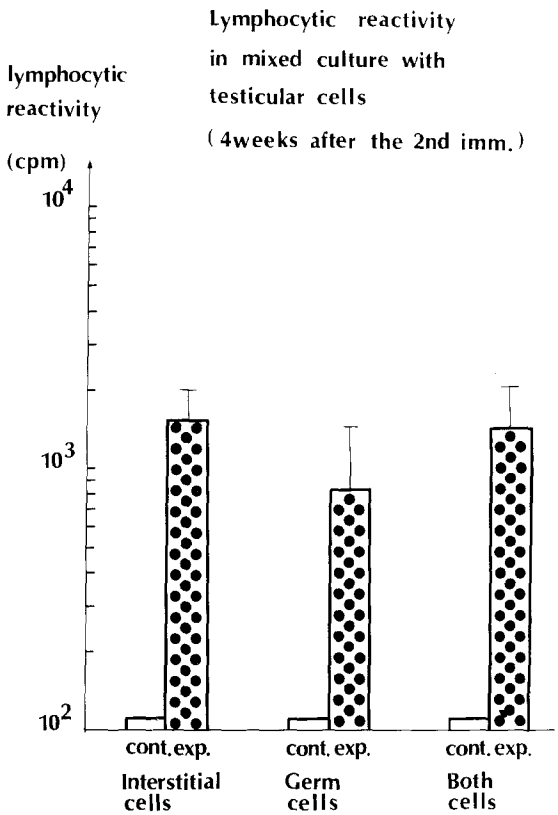

Fig. 13. Lymphocytic reactivity in mixed culture with testicular cells ("interstitial cells", "germ cells", "both cells") at 4 weeks after the 2nd immunization. Significantly positive response of the lymphocytic reactivity against "germ cells" and "both cells", as well as "interstitial cells". The content of germ cells was 40% in "interstitial cells", 65% in "both cells", and 90% in "germ cells". A significant change in the positive response was not observed with the change of germ cell content

g) Kinetics of Histological Changes in Experimental Allergic Orchitis. The histological changes were analyzed quantitatively, in terms of infiltration of lymphomonocytes, infiltration of polymorphs, dilated seminiferous tubules, vacuolar formation in the seminiferous tubules, depression of spermatogenesis, and hyperplasia of Leydig cells. Points from 0 to 3 were given to the severity of the changes of the testes at various stages, and mean values for each change were obtained from several mice at the same stage (shown in Fig. 12). In summary an acute inflammatory reaction, composed of an infiltration of lymphocytes and polymorphs, appeared in the initial phase. The polymorphic reaction ceased up to 4 weeks after the 2nd immunization. A chronic inflammatory reaction composed predominantly of lymphoplasmocytic infiltration, peaked at 4 weeks and ceased at 8 weeks after the 2nd immunization. However, depression of spermatogenesis, vacuolar formation in the tubular lumen, and hyperplasia of Leydig cells peaked at 4 weeks and persisted thereafter as long as 5 months

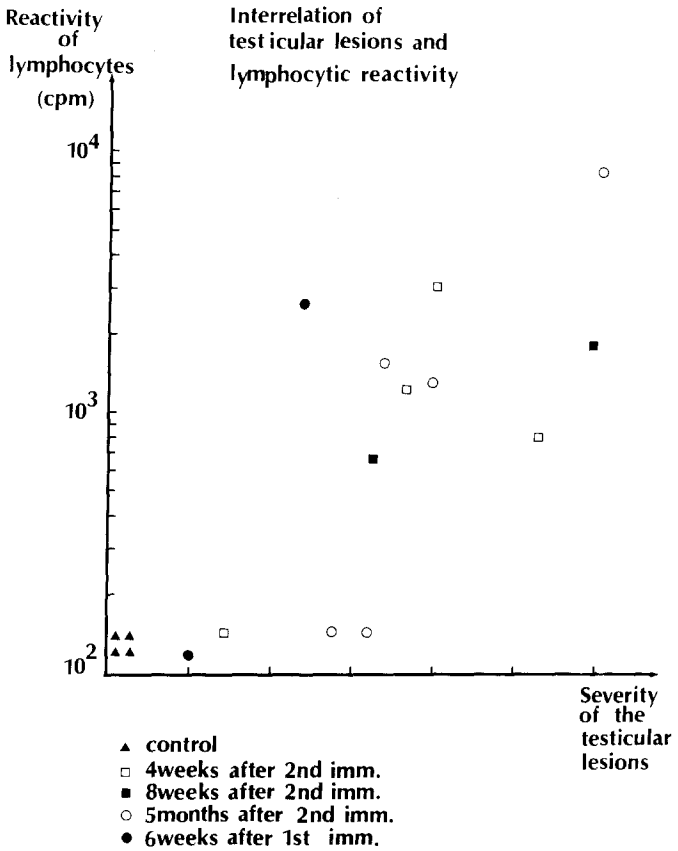

Fig. 14. Interrelation of severity of the testicular lesions and lymphocytic reactivity in mixed culture with testicular cells ("both cells") at various stages. This figure showed the positive correlation between the responsiveness of lymphocytes and the degree of the testicular lesions

after the 2nd immunization. The changes in the testes mentioned above were noticed in over 90% of the all mice in experimental groups.

2. Mixed Lymphocytes Culture with Syngeneic Testicular Cells

Spleen cells or lymph nodes cells, obtained from BAF_1 mice at 6 weeks after the 1st immunization, and 4 weeks, 8 weeks, and 5 months after the 2nd immunization, were cocultivated with syngeneic testicular cells.

The results showed a significantly positive response when lymphocytes from an experimental group were cocultivated with either interstitial cells, germ cells, or both cells (Fig. 13).

In Fig. 14, the degree of the responsiveness of the lymphocytes against both cells were plotted against the degree of the testicular lesions. The figure showed clearly that the higher the responsiveness of lymphocytes, the greater the severity of the testicular lesions. It is interesting to note that lymphocytes obtained from those mice having little inflammatory lesions in the testes also showed positive response against syngeneic testicular cells.

Discussion

There have so far been several reports on systemic histological observations in experimental allergic orchitis in guinea pigs (Freund et al. 1953; Waksman 1959; Brown et al. 1963; Tung 1970). The present paper is the first to report a similar study in mice.

Histological changes in mice were basically similar to those in the guinea pig; i.e., there was an inflammatory reaction, degeneration of germ cells, hyperplasia of Leydig cells, atrophic seminiferous tubules lined by only Sertoli cells, and extensive depression of spermatogenesis, although the onset and severity of these changes differ in each report. One major difference in the orchitis between guinea pigs and mice was that single immunization was enough for the induction of the lesion in the former, while at least two immunizations were required for the induction of overt orchitis in mice. Murine experimental allergic orchitis induced in this way resulted in severe depression of spermatogenesis, which lasted as long as 5 months.

After the third immunization, the severity of the induced orchitis was further enhanced, showing diffuse and extensive inflammation. It is therefore possible to induce experimental allergic orchitis of varying degree in mice, depending on the number of the immunizations.

Testicular lesions from patients suffering from idiopathic infertility are somewhat similar to those observed in murine experimental allergic orchitis at the 5 months after the 2nd immunization; i.e., atrophic seminiferous tubules intermingled with those lined only with Sertoli cells, hyperplastic Leydig cells, occasional mild lymphocytic infiltration, and scattered fibrous scars in the testis. Therefore it has been suggested that some sort of autoimmune process might precede aspermatogenesis in human cases of infertility.

There are two confilicting views concerning pathogenesis of experimental allergic orchitis. One is that the primary testicular lesion is a degeneration of germ cells caused by humoral autoantibody and the inflammation occurs secondarily around the degenerating cells in affected tubules. (Freund et al. 1953, 1955; Brown et al. 1963; Pokorná et al. 1963; Baum et al. 1961). This view is compatible with reports that experimental allergic orchitis can be transferred by the injection of serum from guinea pigs with experimental orchitis (Pokorná et al. 1970; Nagano et al. 1973; Toullet 1976).

The other is that the immune inflammatory reaction by lymphocytes is the primary event, resulting in secondary degeneration of germ cells and advanced aspermatogenesis (Waksman 1959; Levine 1970; Hojo et al. 1980). The latter view is consistent with reports that experimental allergic orchitis can be transferred by the injection of immune cells in guinea pigs (Tung et al. 1971a, 1977; Kantor et al. 1972; Carlo et al. 1976), and mice (Bernard et al. 1978), and that lymphocytes from guinea pig suffering from experimental allergic orchitis showed a positive response in mixed culture with testicular antigen (Hojo et al. 1980) or sperm (Muir et al. 1977).

The present study showed that experimental allergic orchitis started with an initial phase of lympho-monocytic infiltration around the seminiferous tubules, followed by secondary degeneration and destruction of the tubules. In the mixed culture of testicular cells and spleen cells, Hurtenbach et al. (1980) showed that spleen cells from untreated mice were stimulated with interstitial cells, but not with germ cells derived from the immunologically privileged site. The present data showed that lymphocytes from mice immunized with

testicular antigens were stimulated with interstitial cells and germ cells, or mixtures of both. In other words, lymphocytes from orchitis-positive mice showed a significantly higher response in the mixed culture with syngeneic testicular cells, and the magnitude of the responsiveness was well correlated with the severity of the testicular lesions. These findings strongly suggest that cell mediated immunity plays an important role in the induction of experimental allergic orchitis in mice, although a role for humoral autoantibody cannot be ruled out in the present study. In fact, there are several reports suggesting that both cellular and humoral immunity may participate in the induction of experimental allergic orchitis (Brown et al. 1967, 1969; Yantorno et al. 1971; Willson et al. 1972).

Another problem to be resolved is the site of leakage of germ cell antigen, where the immune reaction could occur initially in the testes of immunized mice.

A normal seminiferous tubule is provided with a blood-testis barrier and is well guarded against the entry of autoantibody or immune lymphocyte. There are two circumstances where destruction of the blood-testis barrier is induced, from our results.

First, the selective desquamation and degeneration of spermatogonia and Sertoli cells in the limited area of the tubules (Fig. 1) is assumed to be caused by local ischaemia for which vasospasm was probably responsible. Such cell degeneration might take place as a result of severe damage of the basal lamina of the tubule and is clearly distinct from the preferential degeneration of mature germ cells caused by other agents; X-ray, drug, and hormonal intervention etc..

Second, it is known that obliteration of vasa efferentes in the rete testis brings about obstruction of intratubular flow, eventually leading to germ cell degeneration and loosening of the blood-testis barrier (Smith 1962; Neaves 1973). Other researchers (Johnson 1972) claim that autoantibody entering through the rete testis backflows into the seminiferous tubules. In fact, the rete testis is easily involved in an immune reaction because its wall may be permeable to serum protein (Tung et al. 1971b; Johnson 1972). In this respect, it is not surprising to find frequent occurrence of inflammation of the rete testis in most cases 2 weeks after the 1st antigenic injection. However, the explanation of antibody backflow to the seminiferous tubules is unlikely, since early degenerative changes were observed, mainly in the subcapsular peripheral region. In any event, weakening of blood-testis barrier of the seminiferous tubules caused by alleged vasopsasm, or obstruction of the sperm passage might lead to immunological reactions against the leaked antigens. There remains much to be explored, and a study from a serological viewpoint is now in progress.

Acknowledgements. The authors are extremely grateful to Dr. Y. Tanaka, Director, Department of Pathology, Tokyo Metropolitan Institute of Gerontology, for his invaluable support, to Dr. H. Nariuchi, Associate Professor, Institute of Medical Science, University of Tokyo, for his kindness to offer mice, to Dr. T. Makinodan, Director, Geriatric Center, Wadsworth Medical Center, Los Angeles, for his support.

We thank Mr. H. Gotoh and Miss S. Kubo for their expert assistant techniques.

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Accepted March 18, 1981