The Effects of Adjuvants on Autoimmune Responses Against Testicular Antigens in Mice

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Abstract. Experimental autoimmune orchitis (EAO) is a model of immunologic male infertility and pathologically characterized by lymphocytic inflammation, which causes breakdown of the testicular immune privilege with spermatogenic disturbance. Generally, murine EAO is induced by immunization with testicular homogenate (TH) from the testes of donor mice + complete Freund's adjuvant (CFA) + Bordetella pertussigens (BP), and it has been considered that treatment with these two adjuvants is required to enhance the immune response against testicular antigens. However, there remains a possibility that CFA and BP may affect autoimmune responses against the testicular antigens without TH. In the present study, we examined this possibility using real-time RT-PCR, Western blotting and immunohistochemical staining. The results demonstrated that immunization with TH+CFA+BP evoked more severe EAO than that with only TH. Real-time RT-PCR analyses revealed that Fas mRNA expression in TH+CFA+BP-induced EAO was significantly higher than that in TH-induced EAO. Interestingly, IL-6 mRNA expression dramatically increased in TH+CFA+BP-induced EAO; however, no apparent change in IL-6 mRNA expression occurred in TH-induced EAO. It was also noted that treatment with CFA and BP alone augmented autoimmune reactions against some testicular autoantigens. These results indicate that these adjuvants are helpful in evoking severe EAO, and treatment with the adjuvants alone can evoke autoimmune reactions against some testicular autoantigens despite the use of no TH.

Key words: Adjuvant, Autoimmunity, Testis

In the testes, haploid germ cells (i.e., spermatids and spermatozoa) do not appear in the seminiferous epithelium until puberty, when immune tolerance has already been established. Therefore, they contain various autoimmunogenic materials that are recognized as foreign (non-self) by the immune system. A subcutaneous injection of testicular antigens can induce systemic immune responses against autoantigens of haploid germ cells [1]. However, the testes are immunologically privileged organs. In particular, the blood--testis barrier (BTB) formed by Sertoli cells separates autoimmunogenic spermatozoa from the self immune system [2, 3]. In addition, testicular macrophages and Leydig cells have been found to act as immune suppressors [4].

To overcome the testicular immune privilege, experimental autoimmune orchitis (EAO), a model of immunological male infertility, has been induced by immunization with testicular homogenate (TH) in complete Freund’s adjuvant (CFA) and subsequent intravenous injections of Bordetella pertussigens (BP) in mice and rats [5–7]. EAO is accompanied by epididymo-vasitis and considered to be organ specific because mice injected with CFA+BP+ liver homogenate do not develop inflammation [5, 6]. On the other hand, we established an EAO model induced by two immunizations with syngeneic testicular germ cells or TH alone in mice with a very high incidence [8, 9]. This model is unique in that CFA and BP are not necessary for EAO induction and epididymo-vasitis is hardly observed [8, 9]. In both TH+CFA+BP- and TH-induced EAO, inflammation is Th1 CD4+ cell dependent and involved in secretion of various cytokines and autoantibodies against testicular antigens, which causes damage to seminiferous tubules, namely, sloughing and apoptosis of germ cells [9, 10]. However, there has been no report focusing on the effects of CFA and BP on autoimmune responses against testicular antigens.

In our previous study, we found that BP treatment alone induced systemic leukocytosis in mice with significant pathological changes in the ductuli efferentes, epididymis and prostate, but not in the testes [11]. The aim of the present study was to investigate the effects of CFA and BP on autoimmune responses against testicular antigens using real-time RT-PCR, Western blotting and immunostaining.

Materials and Methods

Animals

A/J mice (aged 8 weeks, n = 43) were purchased from Japan SLIC (Shizuoka, Japan) and housed at the Laboratory Animal Center of Tokyo Medical University for 2 weeks before use. They were maintained at 22–24 C and 50–60% relative humidity with a 12 h light–dark cycle. Approval from the Tokyo Medical University Animal Committee (s-22020) was obtained for this study.

Experimental design

The 10-week-old mice were divided into four groups (one control group and three experimental groups) as follows: (a) Control group
(n = 8), in which the mice were subcutaneously injected with 100 µl of phosphate-buffered saline (PBS) on days 0 and 14; (b) TH group (n = 8), in which the mice were subcutaneously injected with TH obtained from a testis of donor mice (n = 4) in 100 µl of PBS on days 0 and 14; (c) TH+CFA+BP group (n = 8), in which the mice were injected subcutaneously with 100 µl of PBS emulsified with an equal volume of CFA (Sigma–Aldrich, St Louis, MO, USA) immediately followed by intravenous injection of 100 µl of BP solution (2 × 10^10 dead microorganisms/animal, Wako, Osaka, Japan) on days 0 and 14; and (d) CFA+BP group (n = 8), in which the mice were injected subcutaneously with 100 µl of PBS emulsified with an equal volume of CFA followed immediately by intravenous injection of 100 µl of BP solution (2 × 10^10 dead microorganisms/animal) on days 0 and 14. TH was prepared by homogenizing fresh decapsulated testes by ultrasonication for 5 min on ice. The amount of CFA and BP was based on the methods described by Kohno et al. [6]. On day 80, the mice were anesthetized with pentobarbital, and blood was collected from all the mice by cardiac puncture. Serum samples from individual mice were stored at −80°C until assayed. The testes were immediately removed from the sacrificed mice for histological and genetic examination.

**Histological procedure**

The right testes from each mouse of the four groups (n = 8) were examined. The testes were fixed with Bouin’s solution and embedded in plastic (Technovit 7100; Kulzer & Co., Wehrheim, Germany) without cutting the organs to avoid artificial damage to the testicular tissue. Sections (3–4 µm) were obtained at 15–20-µm intervals and stained with Gill’s hematoxylin III and 2% eosin Y for observation by light microscopy (200× magnification). Histopathological changes in spermatogenesis were evaluated using Johnsen’s scoring system [12]. Briefly, scoring was as follows: 10) complete spermatogenesis with many spermatozoa, determined by head form, and an organized germinal epithelium of regular thickness, leaving an open lumen; 9) many spermatozoa present, but with a disorganized germinal epithelium and marked sloughing or obliteration of the lumen; 8) only a few spermatozoa present; 7) no spermatozoa, but many spermatids present; 6) no spermatozoa and only a few spermatids present; 5) no spermatozoa and no spermatids, but several or many spermatocytes present; 4) only a few spermatocytes (<5), but no spermatids or spermatozoa present; 3) spermatogonia were the only germ cells present; 2) no germ cells, but Sertoli cells were present; and 1) no cells in a tubular section. Twenty 1-mm² areas were randomly examined, and more than 200 round- or oval-shaped seminiferous tubules were counted in each testis.

**Tdt-mediated dUTP nick-end labeling (TUNEL) staining**

The left testes from each mouse of the four groups (n = 4) were examined. The testes were fixed in 10% buffered formaldehyde for 3 days. After dehydoration with ethanol, the testes were embedded in paraffin and 4-µm-thick sections were prepared. A commercially available kit (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit; EMD Millipore, Billerica, MA, USA) was used to detect the 3’-OH ends of the DNA strands. Deparaffinized sections were treated with proteinase K (Dako, CA, USA) for 15 min at room temperature (RT) and then washed in distilled water for 4 min. Endogenous peroxidase activity was blocked by treating the sections with 3% H₂O₂ in PBS for 5 min at RT. The sections were incubated in a mixture of terminal deoxynucleotidyl transferase and digoxigenin-labeled deoxyxynucleotides in a humidified chamber at 37°C for 1 h. After reacting with a stop buffer (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit; EMD Millipore) for 10 min, the sections were incubated with an anti-digoxigenin peroxidase conjugate for 30 min. Peroxidase activity was detected by exposing the sections to a solution containing 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB). Negative controls were treated with distilled water in place of the TdT enzyme. For statistical analysis, more than 100 round- or oval-shaped seminiferous tubules were examined, and the number of TUNEL-positive germ cells per seminiferous tubule (mean ± SD) was determined in each mouse. Stained sections were counterstained with methyl green (Vector Laboratories, Burlingame, CA, USA).

**Gene expression analysis**

The left testes from each mouse of the four groups (n = 4) were examined. Total RNA was isolated from the testis using a TRIzol RNA extraction kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, and RNA pellets were dissolved in 10 µl of RNase-free distilled water. Total RNA was measured at 260/280 nm using a UV spectrophotometer and was stored at −80°C prior to use. cDNA was prepared from 10 µg of total RNA in a 100-µl reaction mixture using random primers according to a standard protocol (High-Capacity cDNA Archive Kit; PE Applied Biosystems, Foster City, CA, USA). The PCR reactions were performed in an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA), and the mixtures were stored at −80°C before analysis. Real-time RT-PCR was performed on 3 ng of cDNA using a validated SYBR Green gene expression assay in combination with SYBR Premix Ex Taq II (TaKaRa, Bio, Ohtsu, Japan) for measuring murine Fas, Fas-L, IFN-γ, TNF-α, IL-6, IL-10 and GAPDH. All primers used in this study are listed in Table 1. Th1 cells (involved in delayed-type hypersensitivity) produce TNF-α and IFN-γ, whereas Th2-cells (involved in humoral immunity) produce IL-6 and IL-10 [13, 14]. Apoptosis is one of the main features characterizing germ cell death in the testes and is mediated by the Fas/Fas-L systems [15, 16]. Quantitative real-time PCR was performed in duplicate in a TP800 Thermal Cycler Dice Real Time System (TaKaRa), and the comparative Ct method (2ΔΔCt) was used to quantify gene expression levels. Data of the real-time PCR products were standardized to GAPDH, which was used as the internal control. To confirm the specific amplification of the target genes, each gene product was further separated on 1.5% agarose gel to detect any single bands and the theoretical product sizes.

**Protein isolation and Western blotting analysis**

The right testes obtained from normal mice (n = 3) were homogenized in lysis buffer containing 10 mM of phosphate buffer (pH 7.2), 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml of leupeptin and 1 µg/ml of chymostatin. Protein concentrations were determined by the Bradford method using BSA as a standard. Samples were boiled for 3 min in 0.125 M Tris–HCl, 10% 2-mercaptoethanol, 4% sodium dodecyl sulfate (SDS), 0.004% bromophenol blue and
Immunohistochemical examination

For the detection of serum autoantibodies, the left testes of the normal mice (n = 3) were placed in OCT compound (Miles Laboratories, IL, USA), frozen in liquid nitrogen and stored at −80°C until used. Sections (6 µm) were cut with a cryostat (CM1900; Leica, Wetzlar, Germany), dried in air, fixed in 95% ethanol for 10 min at −20°C, rinsed in air, fixed in 95% ethanol for 10 min at −20°C, rinsed in PBS and then incubated with 50-fold serial dilutions of the collected serum sample for 60 min at RT. After washing in PBS–TWEEN, the blotted membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:500 dilution; ZyMax, Leica, Wetzlar, Germany), dried in air, fixed in 95% ethanol for 10 min at −20°C, rinsed in PBS and then incubated with 50-fold serial dilutions of the collected serum sample for 60 min at RT. After washing in PBS–TWEEN, the blotted membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:500 dilution; ZyMax, Buckinghamshire, UK) in PBS–TWEEN at RT for 1 h. Thereafter, the membranes were incubated with each collected serum sample (diluted 1:100) in PBS–TWEEN at 4°C overnight. After washing in PBS–TWEEN, the blotted membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:10000; Amersham Biosciences, Buckinghamshire, UK) in PBS–TWEEN at RT for 1 h. The membranes were then washed five times with PBS–TWEEN and then examined using the ECL Plus Western Blotting Detection System (GE Healthcare).

Table 1. List of primers used in cloning

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Direction</th>
<th>Sequence 5'-3'</th>
</tr>
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<tbody>
<tr>
<td>Fas</td>
<td>Forward</td>
<td>GCAGACATGCCTGGATCTGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCACAGCAGAGAGATCAGAGA</td>
</tr>
<tr>
<td>Fas-L</td>
<td>Forward</td>
<td>TCCAGGGTGGCTCTACTTAC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCCCCCTTCTCTCTTCTAAGG</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Forward</td>
<td>ATCTGGAGGAACTGGCAAAAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTCAAGACTTCAAAGATGGTGAAG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward</td>
<td>TCTTCTCATTCTCTGTTGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCTACCCAAACTGGATAATACTAG</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>CCAGGAGCTGCTATGGTCAGA</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>GCAGTATGCTTGGATCT GG</td>
</tr>
<tr>
<td>IL-10</td>
<td>Forward</td>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTCCAGCTGGTCCTTTGGTTT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Reverse</td>
<td>TTGCTGTGGAAGTCGACAGG</td>
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10% sucrose and then electrophoretically separated on a 7.5% gradient gel (ATTO, Tokyo, Japan) with 50 µg protein per sample lane. Precision Plus Protein Standards (Bio-Rad Laboratories) were used as molecular mass markers. After electrophoresis, the proteins were electroblotted to polyvinylidene fluoride membranes (Immobilon-P Transfer Membranes; ATTO Corporation). After rinsing in PBS–TWEEN (PBS, 0.1% Tween-20), nonspecific binding was blocked by incubation of the blotted membranes in PBS–TWEEN containing 3% BSA (Sigma–Aldrich) for 1 h at RT. Thereafter, the membranes were incubated with each collected serum sample (diluted 1:100) in PBS–TWEEN at 4°C overnight. After washing in PBS–TWEEN, the blotted membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (ECL) (diluted 1:10000; Amersham Biosciences, Buckinghamshire, UK) in PBS–TWEEN at 4°C overnight. The membranes were then washed five times with PBS–TWEEN and then examined using the ECL Plus Western Blotting Detection System (GE Healthcare).

Immunochemical examination

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Data analysis

Data were expressed as means ± standard deviation (SD), and ANOVA followed by a Tukey-Kramer post hoc test was employed for statistical analysis. A P-value < 0.05 was considered statistically significant.

Results

In the control group, no lymphocytes were observed in the testes (Fig. 1A (a)). However, lymphocytic infiltration with spermatogenic disturbance was found in all the mice in the TH and TH+CFA+BP groups (Figs. 1A (b) and (c)). Spermatogenic disturbance in the TH+CFA+BP group was significantly more severe than that in the TH group (Figs. 1B (b) and (c)). Histopathological changes were not observed in the CFA+BP group, as in the control group (Fig. 1A (d)). Immunohistochemically, TUNEL-positive germ cells were occasionally detected along the basement membrane of the seminiferous tubules in the control and CFA+BP groups. In contrast, many TUNEL-positive germ cells were consistently observed inside the seminiferous tubules in both the TH and TH+CFA+BP groups (Fig. 2A). There were significantly more positive cells in the TH+CFA+BP group compared with the TH group (Fig. 2B).

Real-time RT-PCR analyses revealed that Fas mRNA expression in both the TH and TH+CFA+BP groups significantly increased compared with the control group (Fig. 3). Furthermore, Fas expression in the TH+CFA+BP group was significantly higher than that in the TH group (Fig. 3A). In contrast, Fas-L expression did not show significant changes in the TH and TH+CFA+BP groups (Fig. 3A, P > 0.05 by ANOVA). Although no significant difference in IFN-γ, TNF-α and IL-10 expression was observed between the TH and TH+CFA+BP groups, there was a tendency for augmentation of these expressions in the TH+CFA+BP group compared with the TH group. Notably, IL-6 expression dramatically increased in the TH+CFA+BP group, but did not in the TH group (Fig. 3B). In the CFA+BP group, none of the examined mRNA expressions showed any significant changes (Fig. 3A and B).

To identify the testicular antigens that specifically reacted with sera from each group, we performed SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting by reacting the sera with normal murine testicular homogenates (Fig. 4). In the control group, two immunoreactive bands corresponding to approximately 45 and 100 kDa were detected, showing the presence of natural autoantibodies against these two testicular antigens (Fig. 4a). In the three experimental groups (Figs. 4b, c and d), the two natural autoantibodies were more definitely detected, even in the CFA+BP group, despite the use of no TH (Fig. 4d). In the TH+CFA+BP group, there was a tendency for augmentation of these expressions in the TH+CFA+BP group compared with the TH group. Notably, IL-6 expression dramatically increased in the TH+CFA+BP group, but did not in the TH group (Fig. 3B). In the CFA+BP group, none of the examined mRNA expressions showed any significant changes (Fig. 3A and B).

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To identify the autoantibody-reacting sites, we performed immunohistochemical staining by reacting normal testicular sections with sera from each group (Fig. 5a). No significant staining was detected in the control group in reactions between the sera and frozen
sections of testes from the control mice. Autoantibodies against only haploid cells were observed in the TH group (Fig. 5b), while autoantibodies against all components of the seminiferous epithelium and interstitial cells were found in the TH+CFA+BP group (Fig. 5c). In the CFA+BP group, immunostaining was weak; haploid cells and some cells around the basement membrane of seminiferous tubules were stained (Fig. 5d). Four serum samples from each group were examined, and all produced similar results.

**Discussion**

In the present study, we showed that immunization with TH in combination with CFA and BP evoked more severe autoimmune reactions compared with that with only TH in the testes of mice. Furthermore, we also showed that treatment with CFA and BP alone could evoke autoimmune reactions against some testicular autoantigens.

CFA and BP are ordinarily used as adjuvants to augment an immune response [17, 18]. The use of adjuvants is commonly essential in experiments on induction of organ-specific autoimmune diseases such as experimental autoimmune encephalomyelitis, neuritis, uveitis and thyroiditis in mice [19–23]. However, murine EAO can be induced by immunization with only testicular antigens, which contain various autoimmunogenic materials, without any aid of adjuvants. Damage to the BTB in one testis following an infection or trauma induces orchitis in the contralateral testis [24, 25]. Therefore, it is important to determine the effects of adjuvants on testicular autoimmunity. In the present study, immunization with TH in combination with CFA and BP induced more severe EAO compared with that with TH alone. Moreover, Fas mRNA expression in TH+CFA+BP-induced EAO was significantly higher than that in TH-induced EAO. It appears that the adjuvants led to severe apoptosis of germ cells in the TH+CFA+BP-induced EAO. Regarding cytokine-related mRNA expression, there was a tendency for the mRNA expressions of IFN-γ, TNF-α (Th-1-related cytokines) and IL-10 (a Th2 related cytokine) to be augmented in TH+CFA+BP-induced EAO compared with in TH-induced EAO. Interestingly, the IL-6 (Th2 related cytokine) dramatically increased in TH+CFA+BP-induced EAO, while it remained almost unchanged in TH-induced EAO. The in vitro experiments on seminiferous tubule cultures showed that IFN-γ and TNF-α-induced apoptosis of germ cells through the Fas-FasL system [26, 27]. It was also demonstrated that IL-6-induced apoptosis of germ cells [28]. Therefore, our findings suggested a possibility that a combination of both TH and adjuvants augmented cytokine secretions and induced the severe apoptotic death of testicular germ cells.

In other studies, the employment of CFA and BP has proven to be valuable for indirect alteration of the BTB [17, 18]. Further, immunization with TH in combination with CFA and BP induced an autoimmune reaction against germ cells within the BTB (= autoim-
Adjuvants evoke testicular autoimmunity

We also revealed that autoantibodies were detected only against haploid cell antigens in testicular germ cell-induced EAO sera [32]. The present data showed that an additional band of approximately 40 kDa appeared in the Western blot analysis of sera from the CFA+BP group compared with that of sera from the control group (Fig. 4d) and that the autoantibodies could be detected against both the haploid cells and some cells around the basement of seminiferous tubules by immunostaining (Fig. 5d). These results indicate that the adjuvants were helpful in evoking severe autoimmune reactions against testicular antigens and that the adjuvants alone can evoke autoimmune reactions against some testicular autoantigens despite the use of no TH. The results also suggest that treatment with CFA and BP may cause indirect damage to the BTB resulting in leakage of some autoantigens beyond the BTB. Although some researchers have tried to detect target EAO autoantigens using TH+CFA+BP-induced EAO sera, it remains unclear which proteins are the target antigens [33–36]. Autoantigen analyses using the TH group and/or CFA + BP group may contribute to the discovery of the target proteins of EAO.

Acknowledgments

This work was supported by the Japan Society for the Promotion of Science (KAKENHI 24791673 and 23500504). The authors wish to thank Ms M Kitaoka, Mr S Kawata, Ms Y Ogawa and Ms A Abo for excellent secretarial and technical support.

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