Suppression of adjuvant arthritis in rats by boar seminal immunosuppressive fraction

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Abstract

Objective

The effect of the immunosuppressive fraction of boar seminal vesicle fluid (ISF) was tested on the manifestation of adjuvant arthritis (AA) in rats.

Methods

The inhibitory effect of ISF on mitogen-stimulated proliferation of rat lymphocytes was evaluated by immunoassay using bromodeoxyuridine incorporation. Adjuvant arthritis was induced in male Long Evans rats with Mycobacterium butyricum in adjuvant. ISF was administered at the time of the induction of arthritis. At the time of maximal manifestation of the disease, the hind paw swelling and thymus weight were estimated. IgM and IgG in the rat blood sera were quantified by sandwich ELISA. Serum corticosterone was analyzed by radioimmunoassay. Serum NO_2^-/NO_3^- were estimated by diazotation. Serum albumin was measured spectrophotometrically. The expression of IL-6 mRNA in peritoneal macrophages was estimated by dot-blot hybridization.

Results

Treatment of arthritic rats with ISF attenuated hind paw edema. The production of IgG subclasses dropped in ISF-treated AA rats. The thymus mass and serum albumin concentration were partially restored due to the ISF treatment. Serum corticosterone as well as NO_2^-/NO_3^- concentrations were reduced by the ISF effect. The expression of IL-6 in peritoneal macrophages was inhibited in AA rats after ISF treatment.

Conclusion

ISF attenuated the manifestation of AA in rats and mitigated the inflammation. Immunoglobulin production was most probably inhibited by the decreased proliferation of B lymphocytes.

Key words

Seminal immunosuppressor, adjuvant arthritis inhibition, IgM and IgG subclasses, anti-inflammatory effect.

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Introduction

Rheumatoid arthritis (RA) is a systemic disease characterized by chronic joint inflammation, enhanced acute phase response and hypertrophy of the synovial tissue, which lead to bone and joint destruction. General agreement exists that the pathogenic process of RA is mediated by various T cell types infiltrating the synovial sub-lining cell layer and synovial joint fluid (1). Accumulation of T cells in the synovial tissue, their clone association with antigen-presenting cells, activation of B lymphocytes and the following overproduction of immunoglobulins suggest that RA is a typical autoimmune disease (2).

Adjuvant arthritis (AA), a rat model of RA, is developed after an injection of bacteria suspended in adjuvant (3). An excessive immune reaction follows accompanied by a chronic enhancement of corticosterone secretion that is necessary for survival of the animals (4). Several cytokines and chemokines are involved in a AA progress. TNF , IL-1 and IL-6 are linked to the proliferation of synoviocytes (5) leading to synovial hyperplasia. TNF and IL-1 seem to be connected with early inflammatory events, IL-6 with the late phase of AA (6). Furthermore, inflamed synovial membrane and articular chondrocytes produce high levels of NO^{Σ}, which regulates some inflammatory processes (7).

Some seminal proteins have immunosuppressive effects such as impaired generation of cytotoxic T cells, lowered response of B cells to a variety of antigens (8, 9), decreased cytotoxicity of natural killer cells (10) and inactivation of human anti tumor effector cells (11). Recently, we have isolated the immunosuppressive fraction (ISF) from boar seminal vesicle fluid (12). ISF was identified as a tight complex of two major seminal plasma proteins – PSP I and PSP II (13), both of known primary structure. We established ISF immunosuppressive effect on the antibody response to keyhole limpet hemocyanin, boar epididymal spermatozoa and bacteria (14, 15). After intraperitoneal or rectal administration to mice, ISF was detected in white blood cells (WBC). The number of lymphocytes but not granulocytes in blood was decreased by the ISF effect (12). ISF was detected on spleen B cells from ISF-treated mice, as well, and mitogen-stimulated proliferative activity of these cells was significantly lowered. However, ISF was not found on T cells and did not affect mitogen-induced proliferation of T cells (16).

In the present study, we tested the influence of ISF on the progression of AA development.

Materials and methods

Isolation of ISF from boar seminal vesicle fluid

The usual purification procedure was followed, only the reverse phase HPLC was omitted. The fraction with immunosuppressive activity previously tested on porcine and mouse lymphocytes was used in this study (12).

Series 1: ISF action on mitogen-stimulated proliferation of rat splenocytes

Three male Wistar rats were injected intraperitoneally with 300 g of ISF in 0.5 ml PBS on days 0, 1 and 2. Three controls received saline only. On day 5 the animals were sacrificed and their spleens were excised and homogenized. The cell suspension was passed through 110 m mesh and washed 3 times with saline. After centrifugation at 400g for 5 min, the number of cells was adjusted to 2 x 10^6 cells/ml by RPMI-1640 medium (Serva, Heidelberg, Germany) supplemented with 10% fetal calf serum, L-glutamine (2 mmol/l), penicillin (100 iu/ml) and streptomycin (0.1 g/ml). Triplicate cultures of splenocytes with either 5 mg of PWM (Sigma) or 10 g of PHA (Wellcome Laboratories, Dartford, UK) were incubated in 100 l volumes on microtiter plates (Nunc, Raskilde, Denmark) at 37°C for 72 h. Lymphocyte proliferation was determined by bromodeoxyuridine incorporation. The instructionsprovidedwiththekit (Boehringer, Mannheim, Germany) were followed.

Series 2: AA in rats treated with ISF

Male Long Evans rats (bred in the Department of Normal, Pathological

and Clinical Physiology, Third Medical Faculty of Charles University, Prague) 6 to 7 weeks old, weighing about 160 g were used. Four to 5 rats were housed per cage in an animal room with a 12 h light/dark cycle, controlled humidity and temperature. They had free access to water and a standard pellet diet. The animals were treated with accordance with the national law No. 167/1993 on the use of laboratory animals.

Heat-killed *Mycobacterium butyricum* (Difco, MI, USA) was suspended in paraffin oil, saline and Tween 80 (1: 1: 0.08) to the concentration of 5 mg/ml. AA was induced to the animals by a single intra dermal injection of 50 l of the suspension at the base of the tail. The study consisted of three experimental groups. Animals in group 1 (12 rats) received no treatment and served as negative controls. Animals in group 2 (14 rats) were injected with *Myco - bacterium butyricum* suspended in adjuvant to induce AA (positive controls). The animals in group 3 (14 rats) received 8 doses of ISF intraperitoneally (each dose of 3 mg of ISF in 1 ml of sterile PBS per 1 kg of body weight), on days -3, -2, -1 with respect to the day of AA induction (day 0) and then on days 3, 6, 9, 12, 15 and 18. On day 22, the rats were sacrificed by decapitation. Blood samples were collected, and the serum was separated by centrifugation and stored at -20°C. Hind paw swelling was measured volumetrically. The thymus was dissected and weighed. Peritoneal macrophages were harvested by washing the abdominal cavity with sterile saline, followed by pelleting at 800 xg for 10 min. The macrophages were resuspended in sterile saline and centrifuged at 4000 x g for 30 s. The pellets were used for extraction of total RNA.

Quantification of IgM and IgG sub - classes. The concentrations of IgG_1, IgG_{2a}, IgG_{2b} , IgG_{2c} and IgM in the rat sera collected 22 days after the induction of AA were estimated by sandwich ELISA (17). Microtiter wells were coated with 100 l of affinity purified sheep anti-rat IgG or IgM (2 g/ml PBS, Binding Site, Birmingham, UK) and incubated at 4°C overnight. The coated wells were blocked with PBS-Tween (PBS containing 0.1 % Tween 20, 1% bovine serum albumin, 1% bovine serum) for 1 h at 37°C. For the specific immunoglobulin class determinations, the rat sera were diluted 1:10 for IgG_1 and IgG_{2c}, 1:100 for IgG_{2a}, 1:200 for IgG_{2b} and 1:500 for IgM. The plates were incubated at 4°C overnight, washed and covered with affinity purified sheep antibodies to particular rat immunoglobulin subclasses conjugated with horseradish peroxidase diluted 1:3000 at 37°C for 1 h. Bound peroxidase activity was detected using H_2O_2 as a substrate and o-phenylendiamine as a chromophore. The absorbance at 492 nm was measured by the ELISA reader Spectra II (SLT - Labinstruments, Salzburg, Austria).

Each assay was done in duplicate and the concentrations of particular immunoglobulin subclasses were determined using the calibration curves for standard immunoglobulins (Binding Site, UK).

Corticosterone assay. Serum corticosterone was extracted with methylene chloride and analyzed by radioimmunoassay using specific antibodies (Sigma Aldrich, Deisenhofen, Germany) and [1,2,6,7- ^{3}H] - corticosterone (Amersham, Little Chalfont, UK). Free and bound hormone was separated by dextran-coated charcoal.

Serum nitrate assay. Serum NO_2^-/NO_3^- were estimated after the reduction to nitrate followed by diazotation using the Griess' reagent according to the method of Cortas and Wakid (18).

Serum albumin assay. Serum albumin was measured spectrophotometrically using Albumin (BCGSYS 1 BMI Hitachi kit, Boehringer, Germany) based on the formation of albumin bromcresol green complex.

Expression of IL-6 mRNA. IL-6 mRNA was estimated by dot-blot hybridization. Total RNA was extracted from the pelleted peritoneal macrophages by the guanidinium isothiocyanate method using RNAZOL™ (Cinna Biotech, TX, USA). Samples of RNA were denatured in a mixture of 20 x SSC (0.15 M NaCl/0.015 M trissodiumcitrate) and 37% formaldehyde by heating to 60°C for 15 minutes according to White and Bancroft (19). Samples of 10 g were immobilized on Hybond-N^+ membrane (Amersham, UK) using 15x SSC solution in a dot-blot apparatus (BIO-RAD, CA, USA).

The membrane was baked at 80°C for 30 minutes. Hybridization with the random-primed (Prime-a-Gene® System, Promega, WI, USA) cDNA encoding rat IL-6 (provided by Dr. Bryan Spangelo, University of Nevada, Las Vegas, USA) was performed using the Quick-Hyb® hybridization solution (Stratagene Cloning Systems, La Jolla, CA, USA) according to the instruction manual for 2 h. The blot was washed as recommended by the instruction manual and exposed to an X-ray film (Hyperfilm™, Amersham, UK) at -70°C in an autoradiography cassettewith an intensifying screen for 7 days. Then the blot was stripped by 2 washes in 0.1 x SSC/ 0.5% SDS at 95°C for 20 minutes and rehybridized for random-primed cDNA encoding rat muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The probe originally cloned by Fort *at al.* (20) was amplified in the Laboratory for Diabetes and Nutrition IEE SAS Bratislava, Slovak Republic.

The hybridization and autoradiography procedures were performed according to the same protocol as for IL-6. The blot was autoradiographed for 20 h. The autoradiograms were photographed with a digital camera and analyzed by the Electrophoresis Documentation and Analysis System (Eastman Kodak, NY, USA). The integrated intensity was determined for each spot and IL-6 mRNA was expressed in densitometric units as the ratio of IL-6 to GAPDH intensity.

Statistical analysis

The results were analyzed by one way ANOVA followed by Dunn's test. The significance of the differences in concentrations of particular immunoglobulins between experimental and control groups were analyzed by Student's t-test. Values of $p < 0.05$ are considered as statistically significant.

Results

Isolation of ISF

One ml of seminal vesicle fluid yielded 100-200 mg of ISF.

Table I. Effect of intraperitoneal administration of ISF on rat lymphocytes evaluated by an immunoassay system for detection of bromodeoxyuridine incorporation

Treatment	PHA 10 g/ml	% inhibition	PWM 5 g/ml	% inhibition
Saline	0.249 ± 0.074		0.297 ± 0,056	
ISF	0.132 ± 0.047	47%	0.129 ± 0.042	57 %

Data represent the mean ± SD from three different experiments with 3 rats in each group. Lymphocyte proliferation was expressed as incorporation of 5-bromo-2'-deoxyuridine into replicated DNA determined by ELISA using specific monoclonal antibody.

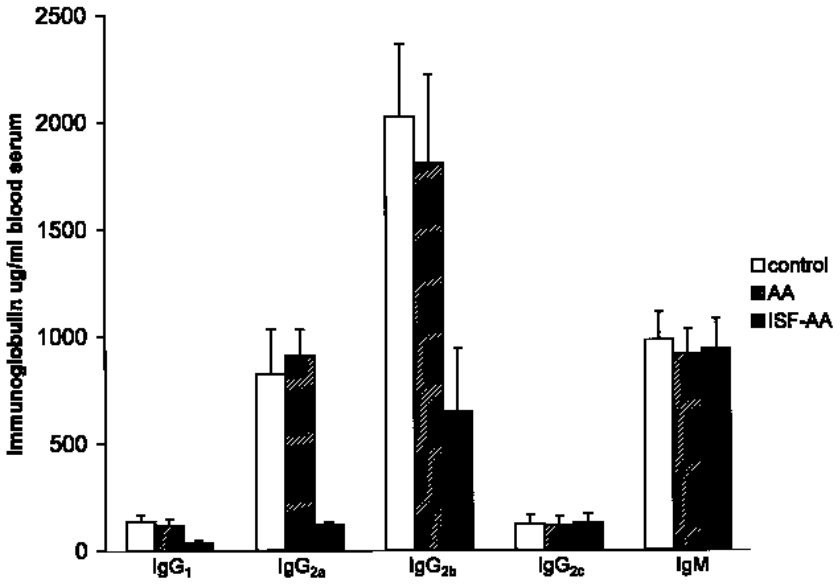

Fig. 1. Effect of ISF on the concentration of IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c} and IgM in rat serum. The results are expressed in mg of immunoglobulins per ml of blood serum evaluated by sandwich ELISA in the sera of naive controls (C),AA rats (AA) and ISF-treated AA rats (ISF-AA). Asterisks represent significance against positive control animals. *p < 0.01.

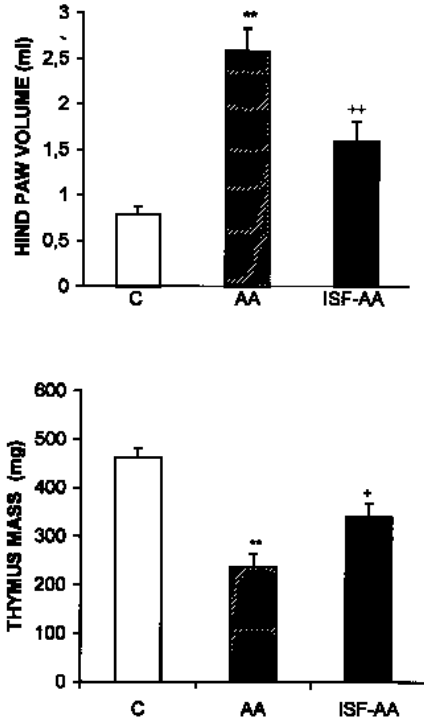

Fig. 2. The upper part depicts the hind paw volumes, the lower part thymus weights of rats on day 22 after inoculation with *Mycobacterium butyricum* suspended in adjuvant. Results are expressed as means of 12 to 14 animals ± S.E.M. **C**: naive controls, **AA**: rats with adjuvant arthritis, **ISF-A**: arthritic rats treated with ISF from day –3 to day 18 of the disease. Asterisks represent significance against C,(**p < 0.01), crosses represent significance against the AA group (++p< 0.01, +p< 0.05).

Series 1. Suppression of mitogen-stimulated proliferation of rat splenocytes by ISF

In splenocytes isolated from ISF-treated animals, the PHA-stimulated proliferative activity was reduced by 47% ($p < 0.01$) and the PWM-stimulated proliferative activity was lowered by 51% ($p < 0.01$) compared to controls (Table I).

Series 2. Effect of ISF on the production of IgM and IgG subclasses in rats with AA

Immunoglobulin concentrations in AA positive controls and naive rats did not differ significantly. However, the production of IgG_1, IgG_{2a} and IgG_{2b} in AA rats treated with ISF dropped dramatically compared with AA animals. No difference between ISF-treated AA rats and AA positive controls was found for IgG_{2c} and IgM concentrations (Fig. 1).

Attenuation of AA manifestation by ISF

Arthritic rats showed the first signs of edema on day 10 of the disease, which fully developed by day 22. In rats treated with ISF the amounts of edema were profoundly reduced, though not totally prevented. The loss of thymus mass observed in arthritic rats was significantly recovered after the administration of ISF (Fig. 2). The serum levels of the negative acute phase reactant albumin were reduced in AA animals and were partially restored by the ISF treatment. Serum nitrate concentrations indicating NO^{Σ} radical production were enhanced in AA rats compared to naive animals, ISF treatment attenuated NO^{Σ} production (Fig. 3). A similar picture was given by the anti-inflammatory hormone corticosterone. High corticosterone levels observed in arthritic rats were suppressed by the ISF treatment. The expression of IL-6 in peritoneal macrophages did not differ between naive and AA animals. However, it was inhibited in AA rats treated with ISF (Fig.4).

Discussion

In this study, the immunosuppressive and anti-inflammatory actions of ISF in the pathogenesis of adjuvant arthritis are presented. The reduced mitogen-stimulated proliferative activity of WBC from rats treated with ISF is in agreement with our previous findings in mice (12) and suggests that the ISF effect is not species specific. ISF seems to impede the development of humoral immune responses by lowering B lymphocyte proliferation and inhibiting the antibody response (16).

Moreover, this study evidenced the abrogated production of immunoglobulin subclasses IgG_1, IgG_{2a} and IgG_{2b} in ISF-treated AA rats. However, IgG_{2c}

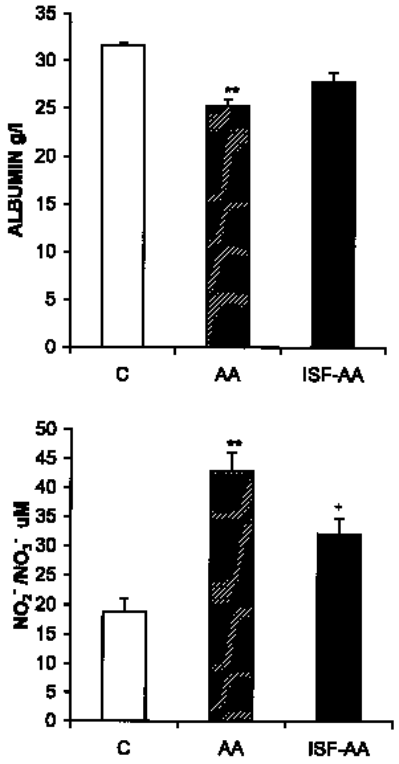

Fig. 3. In the upper panel serum albumin levels are illustrated; in the lower panel serum nitrate levels are shown. For abbreviations see Figure 2 (**p < 0.01, +p < 0.05).

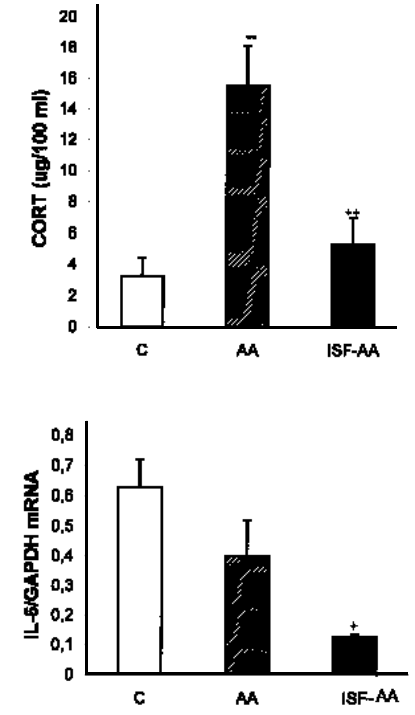

Fig. 4. Serum corticosterone (CORT) levels (upper panel) and concentrations of IL-6 mRNA in peritoneal macrophages (lower panel). For abbreviations see Figure 2 (**p < 0.01, ++p < 0.01, +p < 0.05).

and IgM were resistant to ISF treatment.

ISF deposition partly restored serum albumin levels, which is in line with the suppressed IL-6 expression in peritoneal macrophages in ISF-treated AA rats. As IL-6 is the cytokine accounting for induction of fever and acute phase response (21), its muted expression apparently took part in the reduction of inflammation. It has been shown lately that IL-6 deficient mice were less susceptible to the development of antigen induced arthritis and had remarkably reduced antibody production (22).

Pro-inflammatory cytokines released fromactivated lymphocytes were shown to enhance production of NO^{Σ} radicals (7). We suggest that ISF might reduce NO^{Σ} production by inhibiting the pro-inflammatory cytokines, which play a role in activating the hypothalamo-pituitary-adrenocortical axis. They may stimulate ACTH secretion by a direct action on the pituitary corticotrophs, but their predominant effect is activation of the hypothalamic regulatory neurons triggering secretion of ACTH and corticosterone (23). We assume that ISF attenuated production of pro-inflammatory cytokines that led to the inhibition of the hypothalamic regulatory pathways and lowered the corticosterone release. ISF partially restored arthritis-induced thymolysis.

Our results indicate that ISF inhibits the development of AA in rats. We suppose that the suppression of pro-inflammatory cytokines was followed by a muted acute phase response, NO^{Σ} production, and corticosterone release.

Our previous results indicated that ISF treatment led to prolonged immunosuppression but not to a permanent tolerance to challenging antigens (14). We hypothesize that the prolonged immunosuppression was associated with the continual presence of ISF on WBC membranes. The normal immune response was restored in a relatively short time (weeks) after the ISF application had been ceased. The immunosuppressive fraction of boar seminal plasma may be considered a potent immunosuppressive component with no apparent side effects on long-term treated animals.

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