The Production of Arthritis in Beagles by an Immunological Reaction to Bovine Serum Albumin

Fumihito OHASHI, Terumasa SHIMADA¹⁾, Masahiko SAKURAI, Seiko ISHIHARA, Mitsuru KUWAMURA²⁾, Joji YAMATE²⁾, and Mituaki OTA³⁾

Departments of Veterinary Surgery, ¹⁾Veterinary Teaching Hospital, ²⁾Veterinary Pathology, and ³⁾Veterinary Physiology, College of Agriculture, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 593, Japan

Abstract: Arthritis was produced in beagles by the immunological reaction to bovine serum albumin (BSA). Dogs immunized with BSA showed the development of delayed type-hypersensitivity response to BSA and the significant increase in the titer of serum anti-BSA antibodies. The development of arthritis and the increase in a number of nucleated cells in synovial fluid were observed by the injection with BSA into the knee joints of immunized dogs. The synovial membrane of BSA-injected joints revealed a remarkable villous hyperplasia of membrane, and an infiltration of lymphocytes and plasma cells around vessels, resulting in a lymphoid nodule-like formation. The depositions of IgG and C3 on the surface of the synovial membrane were also observed in BSA-injected joints. Histopathological and immunopathological findings indicated that the immune response to BSA in the knee joints could induce a rheumatoid arthritis like chronic synovitis in dogs.

Key words: arthritis, beagle, bovine serum albumin, immunization

Introduction

Canine rheumatoid arthritis (CRA) is a chronic, progressive, and destructive polyarthritis characterized by remarkable synovitis in the affected joints like human rheumatoid arthritis (HRA) [1, 2, 5, 12, 15]. The synovial histopathological features include a marked villous hypertrophy, a proliferation of superficial synovial cells, and a marked infiltration of chronic inflammatory cells with a tendency to form lymphoid nodules. The chronic progression of this synovitis commonly induced the erosion of cartilage and bone, and finally destroyed the

joints [2].

Although the etiology and pathogenesis of HRA are unclear, the cell-mediated immune response is closely related to the onset of synovitis in this disease [16]. Cook *et al.* [6, 8, 13] reported that immune complexes formed by excessively produced antibodies deposited on the synovial membrane, enhanced the migration and activation of polymorphonuclear leukocytes, and resulted in the progression of synovitis. The etiology and pathogenesis of CRA are also unknown [1, 2]. However, CRA is also considered to be an immunologically-mediated arthritis [2–4, 15], because of the

(Received 2 February 1996 / Accepted 3 March 1996)

Address corresponding: F. OHASHI, Department of Veterinary Surgery, College of Agriculture, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 593, Japan

similarities of clinical and histopathological findings between CRA and HRA.

In this study, the production of arthritis was attempted in beagles by the immunological reaction to bovine serum albumin (BSA) according to Dumonde and Glynn's methods with a slight modification [9]. Delayed type hypersensitivity (DTH) response to BSA, anti BSA antibody titer, clinical scores and the number of nucleated cells in synovial fluid were measured. Histopathological examinations of the synovial membrane and depositions of immune complexes on the surface of joint tissues were also investigated.

Materials and Methods

Animals: Eleven beagles, 4 males and 7 females, were bred in our laboratory. These dogs, at 9 months of age, were divided into two groups: control group (2 males and 3 females) and an immunized group (2 males and 4 females). Administrations of reagents were carried out under xylazine (2 mg/kg) and ketamine (10 mg/kg) anesthesia.

Immunization: BSA (Fraction V; Sigma Chemical Co., St. Louis, U.S.A.) solution, at a concentration of 10 mg/ml in physiological saline, was filtrated by 0.2 μ m sterilized micro filter. The solution was mixed

with an equal volume of Freund's complete adjuvant (Organon Teknika N.V., U.S.A.), enough to form emulsion. The emulsion at a dose of 0.8 ml/kg was injected intracutaneously into the backs and intramuscularly into the femoral regions of the dogs in the immunized group. At 2 and 3 weeks after the first injection, the emulsion was injected again as described above. In dogs of the control group, the emulsion was made from sterile physiological saline and Freund's complete adjuvant, and the injection of emulsion was performed in the same way as that in the immunized group.

Injection with BSA into the knee cavitas articulare: BSA solution, at a concentration of 5 mg/ml in physiological saline, was filtrated by a 0.2 μ m sterilized micro filter. In both groups, 0.3 ml/kg of the BSA solution was injected into the right knee *cavitas articulare* and an equivalent amount of sterile physiological saline was injected into the left knee *cavitas articulare* of each dog, at 1, 2, 3, and 4 weeks after the last immunization (Fig. 1).

Delayed type hypersensitivity (DTH) response: DTH response to antigens well reflects cell-mediated immunoresponse. In both groups, two regions of the dog skin were injected with 0.1 ml of sterilized BSA solution (10 mg/ml in physiological saline) intracuta-

Fig. 1. Changes in anti-BSA antibodies titer in sera (mean $\pm$ SE). *: $P < 0.05$, Mann-Whitney's U test. $\uparrow$: Immunization with BSA. $\blacktriangle$: Injection with BSA into *cavitas articulare*.

neously and one region of the skin was injected with 0.1 ml of sterile physiological saline one week after the last immunization. Twenty-four hr after the injections, the long and short diameters of erythematously indurated regions were measured and the area was estimated.

Serum anti-BSA antibody titer: Five ml of blood was collected from a dog in each group at one week intervals beginning one week after the first immunization. The blood was left for 30 min at room temperature and centrifugalized at $500 \times g$ for 10 min. Sera were separated and stored at -20°C until assayed. The sera were pretreated with heat inactivation for 30 min at 57°C , and the titer of anti-BSA antibodies (IgG subclass) were measured in triplicate by ELISA methods. Briefly, 96-well microtiter plates with flat bottoms were coated with BSA solution (10 $\mu\text{g}/\text{ml}$ in 50 mM sodium carbonate buffer, pH 9.6, standing at 4°C for 16 hr). The plates were washed 3 times with 0.05% Tween 20 in 0.15 M phosphate buffer saline, pH 7.6 (PBS-Tween 20). Hundred μl of 1/2000 diluent of a serum was added per well and incubated for an hour at 37°C . After incubation, the plates were washed 3 times with PBS-Tween 20. Hundred μl of 0.04% o-phenylenediamine substrate solution in citrate-phosphate buffer, pH 5.0 supplemented with 0.03% H_2O_2 were added per well and incubated for 15 min at 37°C . Finally, absorbances were measured with a micro plate reader (Model 450 Micro-plate Reader: Bio-Rad Laboratories, Hercules, U.S.A.) at 495 nm.

Clinical evaluation: During the experimental period, clinical signs and symptoms of arthritis in the control and immunized dogs were evaluated by the modified Goldberg's diagnostic score [11] as follows: 0 points- No or slight clinical sign was observed after BSA-injection into *cavitas articularis*, 1 point- Joint heat and an increase in synovial fluid were observed without lameness, 2 points- Joint heat and an increase in synovial fluid were observed with lameness, 3 points- Joint heat, a marked increase in synovial fluid and an apparent painfulness upon manipulation with intolerance to weight load, 4 points- All clinical signs as "3 points" were observed with joint destruction and dislocation of patella.

Numbers of nucleated cell in synovial fluid: Synovial fluid was aspirated from the right and left knee *cavitas*

articularis of the control and immunized dogs at one week intervals after the last immunization. The aspirated fluid was mixed with an equal volume of hyaluronidase solution supplemented with 10 U/ml sodium heparin for anti-viscosity and anti-coagulation, and then the number of nucleated cells in the fluid was counted with a hemocytometer.

Collection of joint tissues: At the end of the experiments, all dogs were euthanized with sodium pentobarbital. The tissues of synovial membranes were collected from the right and left knee joints of the dogs for histopathological and immunopathological examinations.

Histopathological examination: Tissues were fixed with Lillie's neutral buffered formalin and embedded in paraffin wax. The sections at 3 μm thickness were made and stained with hematoxylin and eosin (HE). Histopathological findings were evaluated by a modified Goldberg's score [11] as follows: 0 points- Synovitis was not apparent. 1 point- Slight proliferation of superficial synovial cells was observed, 2 points- Polytypical synovial membrane and slight infiltration of cells around vessels were observed, 3 points- Apparent synovitis, villous hyperplasia of the synovial membrane, infiltration of lymphocytes and plasma cells and formation of lymphoid nodule-like structures were observed, 4 points- Severe serous and hyperplastic synovitis with marked villous hyperplasia and erosion of the synovial membrane, and formation of lymphoid nodule like structures were observed.

Immunopathological examination: The collected tissues were washed with 0.01 M PBS, pH 7.4, and embedded in O.C.T. compound (Miles Inc., Illinois, U.S.A.). The embedded tissues were frozen by dry ice-aceton and stored at -80°C . The frozen tissues were sectioned into 10 μm thick sections and dried. The dried sections were washed 3 times with PBS for 5 min each time, and drops of optimally-diluted fluorescein isothiocyanate (FITC)-conjugated goat anti-dog IgG antibodies (Bethyl Laboratories, Inc., Montgomery, U.S.A.) or FITC-conjugated goat anti-dog C3 antibodies (The Binding Site LTD., Durham, England) were then added to the sections. After incubation for 40 min at room temperature, the sections were washed 3 times with PBS for 5 min each time and coverslipped with 90% glycerin solution in PBS. The depositions of IgG and C3 in each tissue were investigated with a

fluorescence microscope.

Statistical analyses: Differences between the right and left joints of a dog in the same group were determined by Student's *t* test. Differences between control and immunized groups were evaluated by Mann-Whitney's U-test.

Results

DTH response to BSA: No erythematously indurated region was detected in any of the control dogs. In the immunized group, DTH response to BSA was observed

in 5 of the 6 dogs (83.7%) and the area of indurated regions was $79.1 \pm 17.3 \text{ mm}^2$ (Table 1).

Titer of serum anti-BSA antibodies: In the control group, no anti-BSA antibody was detected until 30 days after the first immunization. The antibody titers were slightly increased after BSA-injection into *cavitas articulare*. In the immunized group, a marked increase in anti-BSA antibody titers was observed after day 11 of immunization, showing a maximum level at day 30 (Fig. 1). The antibody titers in the immunized group were significantly higher than those in the control group at each point examined after day 11 of immunization

Table 1. Delayed type hypersensitivity response to BSA

Group	Positive rate	Area of indurated regions (mm^2)
Control	0/5 (0.0) ^{a)}	— ^{b)}
Immunized	5/6 (83.7)	79.1 ± 17.3 ^{c)}

a): Positive percentage (%). b): not detected; n=5. c): Mean $\pm$ SE; n=6.

Fig. 2. Changes in clinical scores after BSA injection into *cavitas articulare* (mean $\pm$ SE). Injections with physiological saline and BSA into *cavitas articulare* were carried out 4 times weekly. *: $P < 0.05$.

($P < 0.05$).

Clinical signs: Figure 2 shows clinical scores (means $\pm$ SE) after BSA-injection into the knee *cavitas articularis*. In the control group, no marked clinical signs were observed during the experimental period, although one of the 5 dogs revealed a temporary pain in the knee joint after the second BSA-injection. On the other hand, half of the immunized dogs showed pain and swelling of knee joints and lameness after the first BSA-injection, and almost all of the dogs revealed the development and progression of arthritis after the second BSA-injection.

Nucleated cells in synovial fluid: No marked change in the number of nucleated cells in synovial fluid was observed in either knee joints of the control dogs injected with saline in the right and BSA in the left. In addition, no significant difference in cell numbers between the right and left joints in the control group was observed except at 2 weeks after BSA-injection into *cavitas articularis* (Fig. 3a; $P < 0.05$). In the immunized group, no remarkable change in the cell numbers in the fluid of the control (right) joints was detected, whereas a slight increase in the cell numbers in the fluid of the BSA-injected (left) joints was observed at 3 and 4 weeks after BSA-injection (Fig. 3b; $P < 0.05$).

Histopathological findings of synovial membrane: In

the control group, no abnormality of synovial membranes was found in either the control or BSA-injected joints, although some polytypical synovial cells and slight infiltration of cells to the surface of the membrane were observed in a few BSA-injected joints (Fig. 4a).

On the other hand, the synovial membrane of control (right) joints of the immunized group showed polytypical synovial cells and slight infiltration of mononuclear cells around vessels, whereas the membrane of BSA-injected (left) joints revealed the marked villous hyperplasia of the synovial membrane, infiltration of lymphocytes and plasma cells around vessels, and formation of lymphoid nodule-like structures. A remarkable defluxion of synovial cells was also observed in BSA-injected joints (Figs. 4b and c).

The score of histopathological findings are shown in Fig. 5. No significant difference in the scores between the control and BSA-injected joints of control dogs was observed, while the scores of BSA-injected joints of the immunized dogs were significantly higher than those of control joints ($P < 0.05$).

Deposition of IgG and C3: No deposition of IgG and C3 was observed in synovial membranes of either the control or BSA-injected joints in the control group. On

Fig. 3. Changes in numbers of nucleated cells in synovial fluid from control (a) and immunized (b) groups after BSA injection into *cavitas articularis* (mean $\pm$ SE; $n=5$). *: Significant difference between control and BSA injected joints ($P < 0.05$, Student's *t* test).

Fig. 4.

Fig. 5. Histopathological scores of synovial membranes in control and immunized groups (mean $\pm$ SE). *: Significant difference between control and BSA-injected joints ($P < 0.05$).

the other hand, strong depositions of IgG and C3 were observed on the surface of the synovial membrane in the BSA-injected joints of immunized dogs, but not in the control joints (Figs. 6a, b, c and d).

Discussion

Dumonde and Glynn [9] demonstrated that the injection of foreign protein into *cavitas articulare* in rabbits preimmunized with the same alloantigen induced chronic arthritis with infiltration of lymphocytes and plasma cells following acute inflammation 'Arthus reaction' and made the model of rheumatoid arthritis (RA). Several researchers showed that BSA, egg albumin and so on could be used as inducers for RA like chronic arthritis [7, 10, 14]. In this study, BSA, Frac-

tion V, was used as an alloantigen, because it is a beneficial substance in stability and in cost. BSA-Injections into the knee *cavitas articulare* was carried out 4 times at one-week intervals, because, in a preliminary experiment, just one injection as described in Dumonde and Glynn [9] could not induce any clinical and histopathological changes of synovitis in dogs.

DTH responses to BSA could be detected in almost all dogs of the immunized group, but not in the control group. The antibody titer in the immunized group was increased and elevated to a maximum level at day 7 after the last immunization. These immunological features indicate that dogs immunized with BSA had specific cell-mediated and humoral immunity. However, those dogs did not give any clinical signs or histopathological findings of arthritis. Clinical signs and increases in the number of nucleated cells in the synovial fluid were observed after BSA-injections into the knee joints of immunized dogs, demonstrating that BSA-specific immunoresponse might be necessary to induce chronic arthritis.

Gall and Gall [10] demonstrated that the main cells in the synovial fluid of rabbits with BSA-induced arthritis changed from polymorphonuclear cells to lymphocytes at 7 days after BSA-injection into joints. In our study, a remarkable increase in mononuclear cells, lymphocytes and macrophages were also observed at 7 days after BSA-injection in immunized dogs.

Histopathological findings in CRA were villous hyperplasia of the synovial membrane, remarkable infiltration of lymphocytes and plasma cells, formation of lymphoid nodule-like structures and erosion of cartilage and bone [2, 12]. In the present study, CRA-like chronic synovitis without erosion of cartilage and bone was seen in the synovial membrane of BSA-injected joints of immunized dogs. Depositions of immune complexes on the surface of CRA synovial membrane [2, 12] and HRA meniscuses and cartilage [13] were apparent. Immune complexes are well known to be bound to C3. Actually, there were depositions of both IgG and C3 on the surface of the synovial membrane in BSA-injected joints of immunized dogs. Moreover, in these joints, depositions of IgG and C3 were also detected on the surface of meniscuses and cartilage. These histopathological and immunopathological findings indicated that the development of BSA-specific immunoresponses, that is, cell-mediated and humoral

Fig. 4. Synovial membranes in BSA-injected joints of control (a) and immunized (b and c) groups (HE stain). (a): polytypical synovial cells and slightly infiltration of cells on membrane surface ($\times 50$). (b): marked villous hyperplasia of synovial membrane, infiltration of lymphocytes and plasma cells around vessels, and lymphoid nodule-like structures ($\times 50$). (c): lymphoid nodule-like structures with lymphocytes and plasma cells in synovial membrane ($\times 100$).

Fig. 6. Synovial membrane of control and BSA-injected joints in immunized group. Control (a) and BSA-injected joint (b) stained with FITC-conjugated anti-dog IgG. Control (c) and BSA-injected joint (d) stained with FITC-conjugated anti-dog C3. Positive reactions are observed in synovial membrane surface of BSA-injected joint, but not of control joint.

immune responses in the knee joints, could induce CRA-like chronic synovitis in dogs. Finally, a useful model for the investigations of the involvement of local immune responses in CRA and HRA have been made in our study.

References

1. Bennett, D. 1987. Immune-based erosive inflammatory joint disease of the dog: Canine rheumatoid arthritis. 1. Clinical, radiological and laboratory investigation. *J. Small. Anim. Pract.* 28: 779–797.

2. Bennett, D. 1987. Immune-based erosive inflammatory joint disease of the dog: Canine rheumatoid arthritis. 2. Pathological investigations. *J. Small. Anim. Pract.* 28: 799–819.
3. Bennett, L.D. and Kirkham, D. 1987. The laboratory identification of serum rheumatoid factor in the dog. *J. Comp. Path.* 97: 541–550.
4. Chabanne, L., Fournel, C., Faure, R.J., Veyseyre, C.M., Rigal, D., Bringuier, P.J., and Monier, J.C. 1993. IgM and IgA rheumatoid factors in canine polyarthritis. *Vet. Immno. Immunopathol.* 39: 365–379.
5. Clark, D.M. 1991. Current concepts in the treatment of degenerative joint disease. *Compendium.* 13: 1439–1447.
6. Cooke, T.D., Hurd, E.R., Ziff, M., and Jasin, H.E. 1972. The pathogenesis of chronic inflammation in experimental antigen-induced arthritis. *J. Exp. Med.* 135: 323–338.
7. Cooke, T.D. and Jasin, H.E. 1972. The pathogenesis of chronic inflammation experimental antigen-induced arthritis. I. The role of antigen on the local immune response. *Arthritis Rheum.* 15: 327–337.
8. Cooke, T.D. and Richer, S.M. 1974. Pathogenesis of chronic inflammation in experimental antigen-induced arthritis. *J. Rheumatol.* 1: 143–152.
9. Dumonde, D.C. and Glynn, L.E. 1962. The production of arthritis in rabbits by an immunological reaction to fibrin. *Br. J. Exp. Pathol.* 43: 373–383.
10. Gall, E.P. and Gall, E.A. 1980. Histopathogenesis of bovine serum albumin-induced arthritis in the rabbit. *J. Rheumatol.* 7: 13–23.
11. Goldberg, V.M., Lance, E.M., and Davis, P. 1974. Experimental immune synovitis in the rabbit. Relative roles of cells mediated and humoral immunity. *Arthritis and Rheum.* 17: 993–1005.
12. Gorman, N.T. and Werner, L.L. 1986. Immune mediated-diseases of the dog and cat. II. Immune-mediated diseases of the hemolymphatic and musculoskeletal system. *Br. Vet. J.* 142: 403–410.
13. Ohno, O. and Cooke, T.D. 1978. Electron microscopic morphology of immunoglobulin aggregates and their interactions in rheumatoid articular collagenous tissues. *Arthritis Rheum.* 21: 516–527.
14. Phillips, J.M., Kaklamanis, P., and Glynn, L.E. 1966. Experimental arthritis with auto-immunization to inflammatory exudates. *Ann. Rheum. Dis.* 25: 165–174.
15. Romatowski, C. 1984. Comparative therapeutics of canine and human rheumatoid arthritis. *JAVMA* 185: 558–562.
16. Rooney, M., Whelan, A., Feighery, C., and Bresnihan, B. 1989. Changes in lymphocyte infiltration of the synovial membrane and the clinical course of rheumatoid arthritis. *Arthritis Rheum.* 32: 361–369.