Persistent Analgesic Effect of Sustained Release Diclofenac Sodium Preparation on Bovine Type II Collagen-Induced Arthritis

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As a sustained release preparation of diclofenac sodium (DF-Na), we developed a preparation (SR318B) that was expected to exhibit a persistent analgesic effect by once-daily oral administration. In this study, we investigated the persistence of the analgesic effect of SR318B using female cynomolgus monkeys with arthritis induced by sensitization with bovine type II collagen combined with Freund's complete adjuvant. Cynomolgus monkeys in which arthritis was induced to the same severity received oral administration of SR318B (1 mg DF-Na/kg) once daily for 14 days. The mean ellipsoid area of the proximal interphalangeal joints in the monkeys slightly increased before administration on day 3 in the control group. In contrast, the area decreased after day 3 and significantly decreased on day 13 in the SR318B treatment group. The plasma diclofenac (DF) level reached the highest point at 6 hr after administration on day 13, then decreased. The plasma level was lower than the quantification limit 20 hr after administration on both days 0 and 13. In contrast, DF was detected in synovial fluid 24 hr after the initial and final administration. Based on the above findings, SR318B exhibits a persistent analgesic effect on collagen-induced arthritis. While the plasma DF concentration decreased to a level lower than the quantification limit at 20 hr, DF was still detected in the synovial fluid 24 hr after administration. Therefore, the retention of DF in inflammatory regions may play an important role in the analgesic effect.

Key words —— diclofenac sodium, collagen-induced arthritis, sustained release preparation, monkey

INTRODUCTION

A non-steroidal anti-inflammatory drug, diclofenac sodium (DF-Na), exhibits a strong analgesic effect and is used to treat of rheumatoid arthritis, osteoarthritis and lumbago. Sustained release capsules for twice-daily administration were developed by SSP Co., Ltd (Japan).

To reduce adverse effects, such as gastrointestinal disorders, prolong the pharmacological effect, and improve patient compliance, we attempted to develop a preparation for once-daily administration. We designed and prepared four prototype capsules with different enteric properties, and performed pharmacokinetic studies using beagles.[1)] Next, safety and pharmacokinetics were investigated in healthy adult men. Based on the pharmacokinetics of plasma diclofenac (DF), hard capsules containing rapid release granules and enteric film-coated plain granules, combined with an organic acid at a DF-Na ratio of 3:7, were considered to be appropriate as a sustained release preparation for once-daily administration. Oral administration of this sustained release diclofenac sodium preparation (SR318B) exhibited a persistent analgesic effect on a gonitis model prepared by the injection of 2% urate into the knee joint in beagles.[2)]

To confirm the analgesic effect of SR318B, we investigated the persistent therapeutic effect on bovine type II collagen-induced arthritis in monkeys.

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MATERIALS AND METHODS

Materials ——

Test Substance: A SR318B contained 89.98 mg of rapid release granules and 52.68 mg of sustained release granules per capsule, and these contained 7.5 mg and 17.5 mg DF-Na (total of 25 mg), respec-

tively. SR318B was kept in a tightly sealed container and stored at room temperature.

Animals —— Twenty quarantined female cynomolgus monkeys aged 5 years or older were purchased from China National Scientific Instruments & Materials Import/Export Corporation. Animals were acclimatized for 10 weeks before administration of the test substance. Their general condition was observed and food intake was measured daily, body weight was measured on the first and final days of the acclimatization period.

The environmental conditions were 26 ± 2°C room temperature, 50 ± 10% humidity, 15 times/hr ventilation, and 12-hr artificial lighting (6:00–18:00). Animals were kept in individual stainless cages that met the NIH standard. About 108 g of solid food (Teklad Certified 25% Monkey Diet (W), Harlan Sprague Dawley, Inc., U.S.A.) was given at about 15:00, and the leftover was withdrawn on the next day at about 9:00. For drinking water, tap water was given *ad libitum*.

Methods ——

Preparation of Bovine Type II Collagen-Induced Arthritis: Bovine type II collagen was administered to cynomolgus monkeys 2, 5, and 8 weeks after initiation of acclimatization. To 24 mg/vial of bovine type II collagen (COSMOBIO), 6 ml of 5 mmol/l acetic acid-physiological saline was added, and the collagen was dissolved with stirring at 4°C for 24 hr. After this solution was combined with an equal volume of Freund's complete adjuvant (Difco Laboratories), the mixture was mixed very well to prepare the emulsion. This arthritis-inducing substance, 2 ml/head, was intracutaneously injected to cynomolgus monkeys on their backs (1st sensitization). Animals similarly received the second and third administration 3 and 6 weeks after the first sensitization (2nd and 3rd sensitization). Thirteen female cynomolgus monkeys were used in which the development of arthritis was confirmed 10 weeks after initiation of acclimatization (body weight on the day before initiation of test substance administration: 2.20–3.51 kg).

Dose and Administration Method of SR318B: The dose of SR318B was 1.0 mg DF-Na/kg body weight, and SR318B was orally administered once daily for 2 weeks.

In the administration, a capsule was placed deep into the mouth of the monkey using large forceps. Simultaneously, the lower jaw was closed and the common carotid region was rubbed to make the monkey swallow the capsule. After confirming that the monkey swallowed the capsule, about 5 ml of water was poured into the mouth for complete swallowing. Capsules were administered between 21:00 and 22:00.

For experimental groups, a control group consisting of 5 animals received empty capsules (Control), and a SR318B treatment group consisting of 5 animals and a satellite SR318B treatment group consisting of 3 animals (for measurement of plasma and synovial DF concentration) were established. Animals were allocated to obtain the uniform mean ellipsoid area of the proximal interphalangeal joints and mean body weight.

Antibody Titer Measurement: From all animals in the control and SR318B treatment groups, about 1 ml of blood (about 0.4 ml of serum) was collected from the femoral vein once before the administration of bovine type II collagen in the 10th week of acclimatization, and also at 22–24 hr after administration on days 6 and 13 after initiation of administration. The blood samples were kept at room temperature for 40–60 min, then centrifuged at 3000 rpm for 15 min to separate the serum. Using this serum as the primary antibody, the antibody titers were measured by ELISA using goat peroxidase-labeled anti-IgG (Fc) antibody and goat peroxidase-labeled anti-monkey IgM (Fc) antibody as the secondary antibodies.

Measurement of Joint Region: In all animals in the control and SR318B treatment groups, the major and minor axes of the proximal interphalangeal joints of 4 fingers, excluding the thumb, of the front and rear legs were measured using a caliper, and the ellipsoid areas were calculated once before collagen administration, once before SR318B administration, and 22–24 hr after administration on days 3, 6, 10, and 13 of administration. The sum total of ellipsoid areas was regarded as the ellipsoid area per animal. The measurements were performed by the blind method.

Hematologic Test and Blood Biochemical Test: From all animals in the control and SR318B treatment groups, blood samples were collected from the femoral vein once before administration of bovine type II collagen, in the 10th week of acclimatization, and at 22–24 hr after administration on days 6 and 13 after initiation of administration.

Using whole blood treated with an anticoagulant, red blood cell count, white blood cell count, platelet count, hematocrit, and hemoglobin level were measured using a multichannel automatic cell counter (model E-4000, Sysmex Co., Japan). The

ratios of eosinophils, basophils, stab neutrophils, segmented neutrophils, monocytes, and lymphocytes were calculated using a blood cell automatic analyzer (MICROX HEG-120A, Omron Co., Japan). An erythrocyte sedimentation ratio (ESR) was measured by the Westergren method.

The sera obtained by centrifugation were used for a blood biochemical test using an autoanalyzer (Clinalyzer model RX-10, JEOL, Ltd., Japan).

Measurement of Plasma DF Concentration: About 2 ml of blood was collected from the femoral vein using a heparinized syringe in 3 monkeys in the satellite group at 2, 4, 6, 8, 12, 16, 20, and 24 hrs after administration of SR318B on days 0 and 13 (initial and final administration). After being kept on ice, blood samples were centrifuged at 4°C, 3000 rpm for 15 min to obtain plasma. The plasma samples were stored at –20°C, and DF was measured by high performance liquid chromatography.[1)]

Measurement of Synovial DF Concentration: In 3 monkeys in the satellite group, 1 ml of physiological saline was injected into the right knee joint 24 hr after SR318B administration on days 0 and 13 (initial and final administration). The knee was moved, and the synovial fluid was collected. The synovial fluid was stored at –20°C, and DF was measured by high performance liquid chromatography.[1)]

Statistical Analysis: For data on food intake, body weight, ellipsoid area, antibody titer, hematologic test, and blood biochemical test of all the experimental groups, homogeneity was analyzed by the F test. When the variance was homogenous, Student's *t*-test was performed between the control and SR318B treatment groups. When homogeneity was not obtained by F test, the Aspin-Welch test was performed between the control and SR318B treatment groups. Difference of less than 5% were defined as significant.

Since there were individual differences in the severity of arthritis, the ellipsoid areas of the joint regions were examined for outliers by the Dixon method.

RESULTS

The ellipsoid areas of the joint regions were examined for outliers in the control and SR318B groups, and the value exceeded the critical value in one animal in each group. Since joint destruction was observed on X-ray examination at the end of the experiment in these animals, the data were excluded from the results, and the data were evaluated in 4 animals in each group.

Changes in General Condition, Food Intake, and Body Weight

There was no abnormality attributable to SR318B administration other than enlargement of the interphalangeal joints observed after collagen challenge in any animal during the administration period. There were no changes in food intake or body weight attributable to SR318B administration.

Effect of SR318B Administration on Enlargement of Joint Regions

The results of comparison of the ellipsoid area of the proximal interphalangeal joints during the period from before the initiation of SR318B administration until the end of administration are shown in Fig. 1 and Table 1.

In the control group without treatment with SR318B, the mean ellipsoid area of the proximal interphalangeal joints increased to 101.6 ± 1.3% of the value before administration on day 3 of empty capsule administration, showing a slight enlargement of the joint regions, and the area was kept at a similar level until day 13.

In the SR318B treatment group, the mean ellipsoid area decreased after day 3 compared to the value before administration, and the ellipsoid area continued to decrease daily, reaching 89.1 ± 1.2% on day 13. The mean ellipsoid area and the area under the mean ellipsoid area-time curve of the proximal interphalangeal joints were significantly different between the control and SR318B treatment groups on days 10 and 13 of administration.

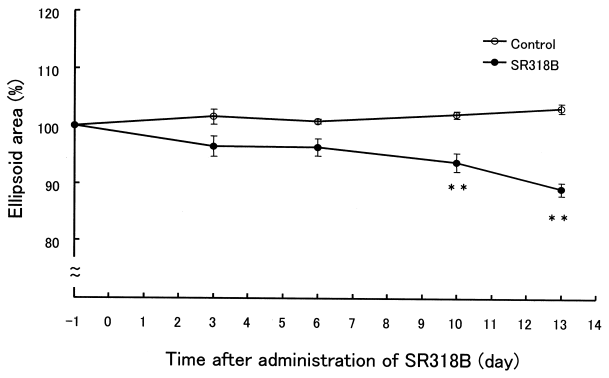

Fig. 1. Effects of SR318B on Bovine Type II Collagen-Induced Arthritis in Female Cynomolgus Monkeys by Measuring the Ellipsoid Area of the Proximal Interphalangeal Joints

Each value represents the mean ± S.E. ($n = 4$). SR318B was orally administered once daily for two weeks. $^{**}p < 0.01$, Significantly different from control group.

Table 1. Effects of SR318B on Bovine Type II Collagen-Induced Arthritis in Female Cynomolgus Monkeys by Measuring the Ellipsoid Area of the Proximal Interphalangeal Joints

Group	Ellipsoid area(%)				
	Time after administration of SR318B				
	Pre	Day 3	Day 6	Day 10	Day 13
Control	100.0±0.0	101.6±1.3	100.9±0.4	102.0±0.6	103.1±0.8
SR318B 1 mg/kg	100.0±0.0	96.5±1.8	96.4±1.5	93.8±1.6**	89.1±1.2**

Each value represents the mean ± S.E. ($n = 4$). SR318B was orally administered once daily for two weeks. $^{**}p < 0.01$, Significantly different from control group.

Table 2. Changes in IgG Antibody Titers during the Period from the Initiation of Sensitization with Bovine Type II Collagen until the End of SR318B Administration in Female Cynomolgus Monkeys

Group	IgG			
	Time after administration of SR318B			
	−10 W	−1 W	Day 6	Day 13
Control	0.284±0.014	1.283±0.521	0.851±0.353	0.845±0.335
SR318B 1 mg/kg	0.297±0.050	0.842±0.049	0.740±0.101	0.721±0.183

Each value represents the mean ± S.E. ($n = 4$). SR318B was orally administered once daily for two weeks.

Table 3. Changes in IgM Antibody Titers during the Period from the Initiation of Sensitization with Bovine Type II Collagen until the End of SR318B Administration in Female Cynomolgus Monkeys

Group	IgM			
	Time after administration of SR318B			
	−10 W	−1 W	Day 6	Day 13
Control	0.234±0.003	0.874±0.243	0.619±0.148	0.619±0.130
SR318B 1 mg/kg	0.246±0.010	1.030±0.089	0.842±0.110	0.753±0.102

Each value represents the mean ± S.E. ($n = 4$). SR318B was orally administered once daily for two weeks.

Changes in Antibody Titer after Collagen Challenge

Changes in IgG and IgM antibody titers, during the period from the initiation of sensitization with bovine types II collagen until the end of SR318B administration, are shown in Tables 2 and 3.

After collagen challenge, both IgG and IgM antibody titers markedly increased in all animals except one (No. 3) in the control group. In the SR318B treatment group, IgG and IgM antibody titers tended to decrease after initiation of administration, but there were no significant differences from those in the control group.

Findings of the Hematologic Test and Blood Biochemical Test

Using blood samples collected before collagen challenge, after collagen challenge (10th week of acclimatization), and on days 6 and 13 of SR318B administration, red blood cell count, white blood cell count, and platelet count were measured.

The ESR increased after collagen challenge in all animals excluding one (No. 3) in the control group. In the SR318B treatment group, ESR tended to decrease after day 6 of administration, and ESR was 29.2% of the value before administration on day 13. In the control group, ESR decreased and returned to the level before administration on day 6 in 2 animals, but ESR decreased to 62.8% of the value before administration on day 13. There were no changes attributable to the administration of SR318B in the other parameters.

In the blood biochemical test, serum samples were analyzed using an autoanalyzer. The measurement items were aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine phosphatase, total bilirubin, total protein, albumin, total cholesterol, triglyceride, glucose, BUN, creatinine, uric acid, inorganic phosphorus, Ca, Na, K, Cl, tartrate-resistant acid phosphatase (TRAP), and C-reactive protein (CRP).

Except for one animal (No. 3) in the control group, CRP increased in all animals after the col-

Table 4. Effects of SR318B in Hematologic and Blood Biochemical Tests on Bovine Type II Collagen-Induced Arthritis in Female Cynomolgus Monkeys

Parameters	Group	Time after administration of SR318B (week)			
		−10 W	−1 W	1 W	2 W
ESR	Control	0.5 ± 0.3	51.3 ± 25.3	19.3 ± 17.6	20.3 ± 18.6
(mm)	SR318B (1 mg/kg)	1.0 ± 0.4	30.8 ± 10.7	17.5 ± 4.7	9.0 ± 3.8
CRP	Control	0.28± 0.28	8.98± 3.07	3.85± 1.36	4.80± 3.29
(mg/dl)	SR318B (1 mg/kg)	0.70± 0.41	8.93± 2.61	8.58± 2.67	6.80± 2.55
ALP	Control	416.3 ± 40.7	1255.0 ±274.1	1137.0 ±258.1	880.3 ±247.5
(IU/l)	SR318B (1 mg/kg)	354.5 ± 77.3	828.8 ±101.3	754.8 ± 96.9	520.3 ± 78.4
TRAP	Control	17.83± 5.48	30.13± 11.39	25.55± 10.91	9.40± 1.81
(IU/l)	SR318B (1 mg/kg)	13.03± 2.33	29.48± 7.40	19.30± 4.10	7.03± 0.74
LDH	Control	1211.0 ±341.5	2030.0 ±648.4	1634.5 ±530.9	571.3 ± 43.7
(IU/l)	SR318B (1 mg/kg)	971.3 ±104.0	2235.5 ±461.6	1481.0 ±238.8	580.8 ± 48.9

Each value represents the mean ± S.E. ($n = 4$). SR318B was orally administered once daily for two weeks. ESR: erythrocyte sedimentation ratio, CRP: C-reactive protein, ALP: alkaline phosphatase, TRAP: tartrate-resistant acid phosphatase, LDH: lactate dehydrogenase.

Table 5. Plasma DF Concentrations after the Administration of SR318B on Bovine Type II Collagen-Induced Arthritis in Female Cynomolgus Monkeys

Dosing period	Plasma DF concentration (μg/ml) Time after administration of SR318B (hr)							
	2	4	6	8	12	16	20	24
Initial	0.01±0.01	0.05±0.03	0.05±0.02	0.04±0.02	0.02±0.01	0.01±0.01	N.D.	N.D.
Final	0.01±0.01	0.04±0.01	0.05±0.01	0.04±0.01	0.02±0.01	0.01±0.01	N.D.	N.D.

N.D.: Not detected (< 0.02 μg/ml). Each value represents the mean ± S.E. ($n = 3$). SR318B (1 mg/kg) was orally administered once daily for two weeks.

lagen challenge. In the SR318B group, CRP decreased to 50 percent on day 6 and returned to the level before administration on day 13. In the control group, CRP decreased on day 13 (Table 4).

ALP and TRAP also increased after the collagen challenge, but the values decreased to 70.1% and 31.2% of the values before administration on day 13, respectively, in the control group. In the SR318B group, ALP and TRAP were 62.8% and 23.8% of the values before administration on day 13, respectively. LDH increased after the collagen challenge, but the value decreased to 26.0% and 28.1% of the values before administration on day 13 in the SR318B treatment group and in the control group, respectively (Table 4).

There were no changes attributable to the administration of SR318B in the other parameters.

Plasma Diclofenac Concentration

The mean plasma DF concentrations after initial administration of SR318B were 0.05 ± 0.03 and 0.05 ± 0.02 μg/ml at 4 and 6 hr after administration, respectively, and the level reached the highest point, 0.05 ± 0.01 μg/ml, 6 hr after administration on day 13 (final administration), then decreased. The plasma concentration was lower than the quantification limit (< 0.02 μg/ml) in two and three of three animals at 16 hr and 20 hr after administration, respectively, on both days 0 and 13 (Table 5).

Measured and Estimated Synovial Diclofenac Concentration

The mean synovial DF concentration 24 hr after the initial and final administration of SR318B were 0.34 ± 0.14 and 0.50 ± 0.11 ng/ml, respectively. These were measured in the sample recovered after 1 ml of physiological saline was injected into the knee joint. The volume of knee joint synovial fluid in cynomolgus monkeys is reported to be about 8 mg. The volume may be 8 μl when the specific gravity of synovial fluid is 1. Assuming that the collected synovial fluid was uniformly diluted with infused physiological saline, we calculated the DF concentration in the actual synovial fluid, and the mean values after the initial and final administration were 0.04 ± 0.02 and 0.06 ± 0.01 μg/ml, respectively (Table 6).

Table 6. Synovial DF Concentrations after Administration of SR318B on Bovine Type II Collagen-Induced Arthritis in Female Cynomolgus Monkeys

Dosing period	Synovial concentration (ng/ml)[a)]	Actual synovial concentration (μg/ml)[b)]
Initial	0.34 ± 0.14	0.04 ± 0.02
Final	0.50 ± 0.11	0.06 ± 0.01

a) These were measured in the sample recovered after 1 ml of physiological saline was injected into the knee joint. *b*) Assuming that the collected synovial fluid was uniformly diluted with infused physiological saline, we calculated the DF concentration in the actual synovial fluid. Each value represents the mean ± S.E. ($n = 3$). SR318B (1 mg/kg) was orally administered once daily for two weeks.

DISCUSSION

Female cynomolgus monkeys with bovine type II collagen-induced arthritis, which present an animal model most similar to human rheumatoid arthritis,[3)] received an oral administration of SR318B once daily for 14 days, and the inflammatory enlargement of the knee joint was measured to investigate the therapeutic effect of SR318B on chronic arthritis. The plasma and synovial diclofenac concentrations were measured in a satellite group that received the same SR318B administration. The control group received empty capsules by the same method as SR318B administration.

In bovine type II collagen-induced arthritis, the proximal interphalangeal joints of the front and rear legs enlarged, IgG and IgM antibody titers increased, ESR increased in a hematologic test, and CRP, ALP, TRAP, and LDH increased in a blood biochemical test. All parameters that increased after sensitization in both the control and drug treatment groups decreased during the SR318B administration period. The decreases were greater in the SR318B treatment group than in the control group, but the differences between the two groups were not significant.

The mean ellipsoid area of the proximal interphalangeal joints slightly increased to $101.6 \pm 1.3\%$ of the value before administration on day 3 of administration in the control group. In contrast, the mean ellipsoid area decreased after day 3 and reached $89.1 \pm 1.2\%$ of the value before administration on day 13 in the SR318B treatment group. There were significant differences in the mean ellipsoid area between the control and SR318B treatment groups on days 10 and 13, suggesting the therapeutic effect of SR318B on collagen-induced arthritis. No abnormalities attributable to SR318B administration were observed in general condition, food intake, changes in body weight, or autopsy at the end of the experimental period.

Changes in the plasma diclofenac concentration after SR318B administration were similar after the initial and final administration, and on both days, the level was below the quantification limit after 20 hr in all animals, showing no increase due to repeated administration. However, DF was detected in the synovial fluid 24 hr after the initial and final administration, and the calculated concentrations were 0.04 ± 0.02 and 0.06 ± 0.01 μg/ml, respectively. The fact that orally administered DF was still detected in the inflammatory region 24 hr after administration may have played an important role in prolonging the analgesic effect of SR318B. The importance of tissue concentration for the effect of nonsteroidal anti-inflammatory drugs has been previously reported.[4,5)]

Based on the above findings, SR318B exhibits a therapeutic effect on arthritis. Although the plasma DF concentration decreased to a level lower than the quantification limit 20 hr after administration, DF was still detected in the synovial fluid 24 hr after administration. Therefore, retention of SR318B in the articular cavity may reflect its persistent therapeutic effect.

REFERENCES

1) Takahashi, M., Horie, T., Kasai, S. and Tezuka, M. (2001) Development of a sustained release preparation of diclofenac sodium and its pharmacokinetics in dogs. *Xenobio. Metabol. and Dispos.*, **16**, 451–460.
2) Takahashi, M., Umehara, N., Suzuki, S. and Tezuka, M. (2001) Analgesic action of a sustained release preparation of diclofenac sodium in a canine urate-induced gonarthritis. *J. Health Sci.*, **47**, 464–467.
3) Ukari, Y., Fujii, K. and Murota, K. (1993) Therapeutic effect of high molecular weight hyaluronate on rheumatoid arthritis: Experimental study on monkey with collagen-induced arthritis. *Jap. J. Rheumat. Joint Surg.*, **12**, 351–366.
4) Nolan, J. C., Osman, M. A., Cheng, L. K. and Sancilio, L. F. (1990) Bromfenac, a new nonsteroidal anti-inflammatory drug: Relationship between the anti-inflammatory and analgesic activity and plasma drug levels in rodents. *J. Pharmacol. Exp. Ther.*, **254**, 104–108.
5) Bendele, A. M., Ruterbories, K. J., Spaethe, S. M., Benslay, D. N., Lindstrom, T. D., Lee, S. J. and

Naismith, R. W. (1992) Correlation of anti-inflammatory activity with peak tissue rather than peak plasma levels of BF389. *J. Pharmacol. Exp. Ther.*, **260**, 1194–1198.