THERAPEUTIC EFFICACY OF BICYCLOMYCIN FOR SHIGELLOSIS EXPERIMENTALLY INDUCED IN RHESUS MONKEYS

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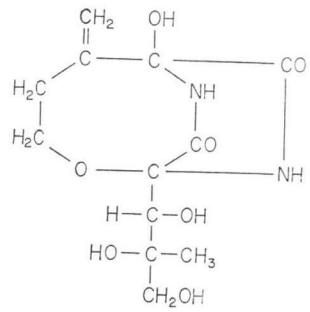
The efficacy of orally given bicyclomycin on shigellosis was studied in rhesus monkeys infected with *Shigella flexneri* 2a. All the animals developed lethal infection after rectal inoculation of the virulent strain 5503. Bicyclomycin and the kanamycin control were given orally at daily dosage of 40 mg/kg for a period of 5 days beginning 24 hours after the inoculation. Bicyclomycin compared favorably with kanamycin in respect to 1) the time required for termination of bloody mucous feces, restoration of normal feces, and disappearance of *Shigella* bacilli from feces and 2) the absence of the bacilli from intestinal tissues at autopsy. This experiment was preceded by a fundamental study, which revealed satisfactory activity of bicyclomycin against various *Shigella* species (MIC: 6.25~25 mcg/ml) and good stability and excretion in feces. These data demonstrate the therapeutic efficacy of bicyclomycin in intestinal infections.

Bicyclomycin, a new antibiotic developed in the Research Laboratories of Fujisawa Pharmaceutical Co., Ltd., has the unique chemical structure shown in Fig. 1[1]. It was isolated from a culture filtrate of *Streptomyces sapporonensis* ATCC 21532[2] and has proven to be highly active against gram-negative organisms except for *Proteus* species and *Pseudomonas aeruginosa*. It is devoid of cross-resistance to any available antibiotics[3], and is poorly absorbed from the gastro-intestinal tract.[4]

Fig. 1. Chemical structure of bicyclomycin.



$C_{12}H_{18}N_2O_7$ M.W. 302.28

The present paper is concerned with the efficacy of orally administered bicyclomycin in experimentally induced shigellosis in rhesus monkeys.

Materials and Methods

1. Bacterial strains

One hundred strains of *Shigella* bacilli used for determination of MIC were isolated at the Osaka Municipal Momoyama Hospital and the Hospital of Kobe University. *Shigella flexneri* 2a, 5503 used for provoking infection in monkeys was provided by Dr. HONJO,

National Institute of Health, Tokyo. The MICs of bicyclomycin and kanamycin for this strain were found to be 25 mcg/ml and 12.5 mcg/ml, respectively, by the agar dilution method.

2. Antibiotics

Antibiotics used were: bicyclomycin (Fujisawa Research Laboratories), kanamycin sulfate (Meiji Seika Co., Ltd.), chloramphenicol (Fujisawa Pharmaceutical Co., Ltd.), tetracycline hydrochloride (Takeda Chemical Industries, Ltd.), streptomycin sulfate (Meiji Seika Co., Ltd.), ampicillin (Beecham Research Laboratories) and nalidixic acid (Daiichi Pharmaceutical Co., Ltd.).

3. Determination of MIC

One loopful of a test strain that had been incubated at 37°C for 20 hours in Trypticase soy broth (BBL) was streaked on Heart infusion agar media containing serial twofold dilutions of antibiotics. Inoculated plates were then incubated at 37°C for 20 hours, and the minimum concentration required for complete inhibition of visible growth was recorded as the MIC.

4. Bioassay

The concentration of bicyclomycin and kanamycin in specimens was determined by the disk method using *Escherichia coli* ATCC 27166 and *Bacillus subtilis* ATCC 6633, respectively, as test organisms. The method is detailed in our preceding paper[4].

5. Stability in feces

Feces of three healthy volunteers were suspended at a concentration of 35 % in sterile distilled water. A 4.5-ml portion of this suspension was mixed with 0.5 ml of a 10,000 mcg/ml bicyclomycin solution, incubated at 37°C for 3 and 8 hours, and diluted to 50 ml. This mixture was then centrifuged, and the potency of bicyclomycin in the supernatant fluid was determined.

6. Fecal and urinary excretion

Five healthy rhesus monkeys were fasted for 24 hours and given a single oral dose of 40 mg/kg of bicyclomycin. The urine and feces were collected separately over three periods; 0~24 hours, 24~48 hours, and 48~72 hours. The feces were suspended in distilled water to a concentration of 20 %. This suspension was centrifuged, and the quantity of antibiotic in the supernatant fluid was determined by the disk method. The five monkeys were given bicyclomycin and kanamycin in a crossover design, and the concentration and recovery of bicyclomycin in the feces and urine were compared with those of kanamycin.

Three healthy fasted volunteers were given a single dose of 1 g of bicyclomycin, and the 0~24 and 24-48 hour urines and feces were assayed in the same manner.

7. Thin-layer chromatography and bioautography of active substance recovered in feces

Healthy volunteers were given 1 g of bicyclomycin, and the feces were suspended in distilled water to give a concentration of 30 %. This suspension was centrifuged, and the supernatant fluid was spotted on a #6060 Eastman Chromatogram sheets (silica gel). The sheet was developed with a 5:1 mixture of chloroform and methanol, air-dried, and placed on an agar plate seeded with *E. coli* ATCC 27166. After removal of the sheet, the agar plate was incubated at 37°C for 20 hours, and the Rf values were determined.

8. Shigellosis experimentally induced in rhesus monkeys

The method described by TAKASAKA *et al.* was used.[5] Nineteen male rhesus monkeys weighing 3.0~5.0 kg were divided into 7-, 5-, and 7-animal groups, fasted for 48 hours, and given 10 ml of physiological saline rectally to eliminate the feces. Under general anesthesia 10 ml of physiological saline containing 1×10^{10}/ml of *Sh. flexneri* 2a, 5503 was infused into the rectum. Within 24 hours all the animals developed bloody mucous feces typical of shigellosis.

Over a period of five days beginning 24 hours after challenge, monkeys of the first 7-animal group were given a single dose of 40 mg/kg of bicyclomycin and those of the 5-animal group the same dose of kanamycin. The remaining 7 animals served as controls and received physiological saline in place of an antibiotic. The period of observation was 3 weeks, and observation was made on the gross appearance of feces, the count of viable

Shigella cells in feces, and the sensitivity of isolated *Shigella* bacilli to the antibiotic.

Autopsy was performed on all the infected animals, and the ileum, cecum, colon, and rectum were checked for the presence of *Shigella* bacilli after the tissues had been smeared on SS agar plates. The isolation and identification of *Shigella flexneri* 2a, 5503 were conducted with SS agar and factor sera.

Results

1. Susceptibility of Freshly Isolated Strains of *Sh. sonnei* and *Sh. flexneri* to Bicyclomycin and Six Other Antimicrobial Agents

The susceptibilities of *Sh. sonnei* and *Sh. flexneri* (50 strains each) are shown in Table 1.

Table 1. Susceptibility of freshly isolated strains of *Sh. sonnei* and *Sh. flexneri* to bicyclomycin and six other chemotherapeutics.

Organism	Antibiotic	MIC: mcg/ml									
		>800	800	400	200	100	50	25	12.5	6.25	3.13
Shigella sonnei (50 strains)	Bicyclomycin						2	34	14		
	Chloramphenicol		47	1	1					1	
	Streptomycin	7	28	12					1	2	
	Tetracycline			18	30			1		1	
	Kanamycin	5						11	30	4	
	Nalidixic acid		1	2		1				7	39
	Ampicillin								5	45	
Shigella flexneri (50 strains)	Bicyclomycin							6	43	1	
	Chloramphenicol		5	9	11						25
	Streptomycin		6	11	1				6	26	
	Tetracycline			2	14			4	5	8	17
	Kanamycin								25	25	
	Nalidixic acid								3	4	43
	Ampicillin	1						2	18	29	

(1) *Sh. sonnei*

All 50 strains were susceptible to bicyclomycin. Bicyclomycin at concentrations of 12.5 and 25 mcg/ml inhibited 48 strains and the remaining 2 strains were inhibited at 50 mcg/ml. Most of the 50 strains were susceptible to kanamycin (MIC: 6.25~25 mcg/ml) and nalidixic acid (MIC: 3.13 and 6.25 mcg/ml) but were highly resistant to chloramphenicol (MIC: 200~800 mcg /ml), streptomycin (MIC: >400 mcg/ml), and tetracycline (MIC: 200 and 400 mcg/ml). All the 50 strains were susceptible to ampicillin.

(2) *Sh. flexneri*

All 50 strains of *Sh. flexneri* were inhibited by 6.25~25 mcg/ml of bicyclomycin. MIC values for chloramphenicol, streptomycin and tetracycline assumed distinct biphasic distribu-

tions: one from 200 to 800 mcg/ml and the other 25 mcg/ml or less. The 50 strains were susceptible to kanamycin (MIC: 3.13～12.5 mcg /ml), nalidixic acid (MIC: 3.13～12.5 mcg/ml) and, except for one strain, to ampicillin (MIC: 6.25～25 mcg/ml).

2. Stability of Bicyclomycin in Suspension of Feces

Feces of three healthy volunteers were suspended in distilled water and incubated with bicyclomycin at 37°C. After 3 and 8 hours, bicyclomycin recovery was 94～98 % indicating high stability in human feces (Table 2).

Table 2. Stability of bicyclomycin in suspension of human feces.

	Residual activity (%)		
	0 hr.	3 hr.	8 hr.
K.K.	98	98	94
K.N.	98	98	95
Y.F.	100	98	98
Control (P.B) pH 5.4	99	95	90

3. Fecal and Urinary Excretion after Oral Administration to Monkeys and Healthy Volunteers

(1) Monkeys

The concentration and recovery of bicyclomycin in feces after a single dose of 40 mg/kg were compared in a crossover design with that of kanamycin in five healthy rhesus monkeys.

The results are shown in Table 3. The concentration in the 72-hour feces ranged from 1,717 to 4,060 mcg/g, *i.e.* 35.0～75.0 % (mean: 46.2 %) of the given dose was excreted in the

Table 3. Fecal and urinary excretion after a single oral dose of 40 mg/kg of bicyclomycin to monkeys.

Monkey		0～24 hr.		24～48 hr.		48～72 hr.		Total	
		mcg/g* or mcg/ml	%	mcg/g*, mcg/ml	%	mcg/g*, mcg/ml	%	%	%
No. 707	Feces*	2,670	42.8	619	2.1	—	—	44.9	47.5
	Urine	34	2.6	—	—	—	—	2.6	
No. 737	Feces	—	—	1,717	35.2	912	3.9	39.1	40.9
	Urine	66	1.8	—	—	—	—	1.8	
No. 731	Feces	4,060	68.7	1,150	6.3	—	—	75.0	79.0
	Urine	57	4.0	—	—	—	—	4.0	
No. 749	Feces	3,110	15.0	1,880	21.8	—	—	36.8	40.3
	Urine	37	3.5	—	—	—	—	3.5	
No. 755	Feces	—	—	1,880	20.0	1,600	15.0	35.0	38.5
	Urine	40	3.5	—	—	—	—	3.5	
Mean	Feces	3,280 (n=3)	42.2	1,450	17.1	1,260 (n=2)	9.5	46.2	49.2
	Urine	47	3.1	—	—	—	—	3.1	

feces during this period. The recovery in the 72-hour urine was as little as 1.8～4.0 % (mean: 3.1 %). Bicyclomycin was thus shown to be absorbed poorly from the gastrointestinal tract and nearly one half the given dose was excreted unchanged in the feces.

Kanamycin was also found to be absorbed poorly from the gastrointestinal tract (Table 4). The rate of recovery in feces and urines in the 72-hour period averaged 28.9 % (16.8～46.4 %) and 2.4 % respectively. Fecal concentration varied from 400 to 9,680 mcg/g and were found to depend on the quantities of excreta. These findings proved that fecal concentrations of bicyclomycin are high enough to kill sensitive microorganisms.

(2) Healthy volunteers

Table 4. Fecal and urinary excretion after a single oral dose of 40 mg/kg of kanamycin to monkeys.

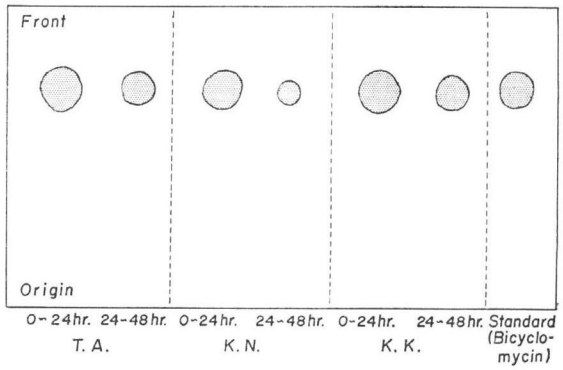
Monkey		0~24 hr.		24~48 hr.		48~72 hr.		Total	
		mcg/g*, mcg/ml	%	mcg/g*, mcg/ml	%	mcg/g*, mcg/ml	%	%	%
No. 707	Feces*	346	5.1	741	14.2	20	0.3	19.7	22.4
	Urine	39	2.1	5.7	0.4	12.6	0.2	2.7	
No. 737	Feces	—	—	842	15.4	519	11.3	26.7	29.3
	Urine	56.5	1.9	3.3	0.2	12.5	0.5	2.6	
No. 731	Feces	252	4.6	400	11.5	70	0.7	16.8	20.6
	Urine	25.5	3.5	2.7	0.3	—	—	3.8	
No. 749	Feces	2,600	13.6	—	—	9,680	21.4	35.0	37.3
	Urine	49	1.5	7.3	0.2	15.0	0.5	2.5	
No. 755	Feces	—	—	540	1.1	3,850	45.3	46.4	46.8
	Urine	3.3	0.2	1.9	0.2	—	—	0.4	
Mean	Feces	1,070	7.8	630	10.6	2,830	15.8	28.9	31.3
	Urine	34.7	1.8	4.2	0.3	13.4	0.4	2.4	

Table 5. Fecal excretion after a single oral dose of 1 g of bicyclomycin to human volunteers.

Volunteer (male)	0~24 hr.			24~48 hr.			Total %
	mg/g	mg	%	mg/g	mg	%	
T.A. 62 kg	7.26	413.8	41.4	1.86	306.6	30.7	72.1
K.N. 72 kg	3.93	621.1	62.1	0.68	156.4	15.6	77.7
K.K. 63 kg	5.46	437.0	43.7	1.10	258.8	25.9	69.6
Mean	5.55	490.6	49.1	1.21	240.6	24.1	73.2

A single dose of 1 g of bicyclomycin was given to three healthy male volunteers, and the concentration and recovery in the 48-hour feces were determined. The results are shown in Table 5. The concentration in the first 24-hour feces averaged 5,550 mcg/g, *i.e.* 49.1 % (mean) of the given dose was excreted during this period. The concentration in the 24- to 48-hour feces averaged 1,210 mcg/g, *i.e.* 24.1 % of the given dose was excreted during this period. Accordingly, the recovery in the 48-hour feces totaled 73.2 %. Thus a single oral dose of 1 g was shown to produce fecal concentrations high enough to kill sensitive organisms in man.

Fig. 2. Bioautograms of feces of volunteers receiving bicyclomycin.
Dose: Bicyclomycin, 1 g/man, p.o.
Solvent: $CHCl_3$-CH_3OH (5:1).
Chromagram sheet: Eastman silica gel 6060.
Test organism: *E. coli* ATCC 27166.



The active substance recovered in human feces was identified as unchanged bicyclomycin, since its bioautogram produced a single inhibition zone having an Rf value identical with that of authentic bicyclomycin (Fig. 2).

4. Effect on Shigellosis in Monkeys

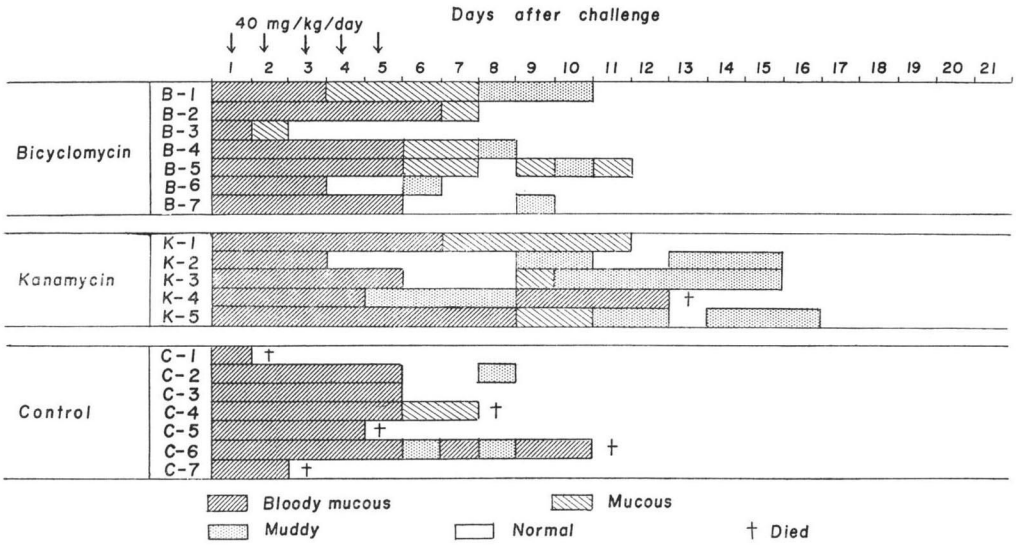
The effect of bicyclomycin on shigellosis of rhesus monkeys was compared with that of kanamycin.

(1) Gross findings of feces

Five of the seven untreated animals died within 11 days. Normal feces in the remaining 2 animals were restored on the 6th and 9th days. As shown in Fig. 3, the 7 animals receiving bicyclomycin responded satisfactorily to 40 mg/kg of this antibiotic given for 5 days and survived the 21-day observation period. Excretion of bloody mucous feces in all subjects terminated in an average of 5±0.7 days, and feces became normal in 8.6±1.1 days.

Of the 5 animals receiving kanamycin, bloody mucus was terminated in the feces of 4 animals in 6.5±1.0 days and the feces returned to normal in 15.3±1.1 days. The remaining one animal (K-4) died on the 12th day.

Fig. 3. Gross findings of feces.



These findings suggest superiority of bicyclomycin over kanamycin from the viewpoint of restoration of normal feces.

(2) Cell count of *Sh. flexneri* in feces

As Fig. 4 shows, the count of bacilli surviving in the feces varied greatly. The period required for complete disappearance of the bacilli averaged 7.0±1.1 days in the bicyclomycin group and 9.3±2.7 days in the kanamycin group. In one animal of the kanamycin group excretion of the bacilli terminated on the 8th day, but returned on the 11th day and continued for 5 days. Animals receiving bicyclomycin suffered no such relapse.

During and after the 5-day treatment the sensitivity of isolated *Sh. flexneri* to bicyclomycin remained the same as the sensitivity at the time of challenge.

3. Isolation of *Sh. flexneri* from Tissue of Ileum, Cecum, Colon, and Rectum at Autopsy

Tissues of the ileum, cecum, colon and rectum were examined for the presence of *Sh. flexneri*. All the animals were autopsied immediately after death or after sacrifice which was performed 20 days after challenge. The results are shown in Fig. 5.

Control (7 animals): *Sh. flexneri* was present in every portion of the intestines of 4 animals and in two or more portions of 2 animals, but was absent in the remaining one

Fig. 4. Count of viable cell of *Sh. flexneri* 2a in feces.

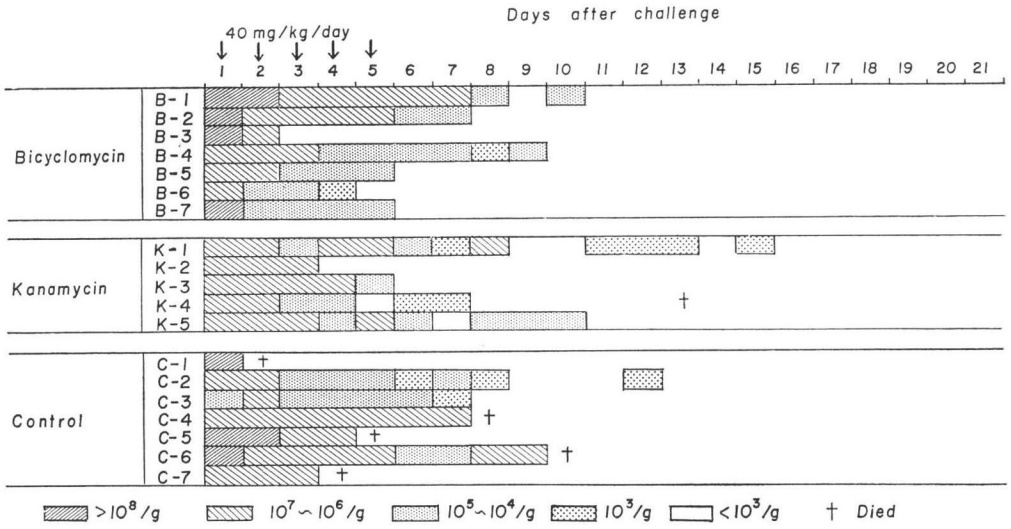
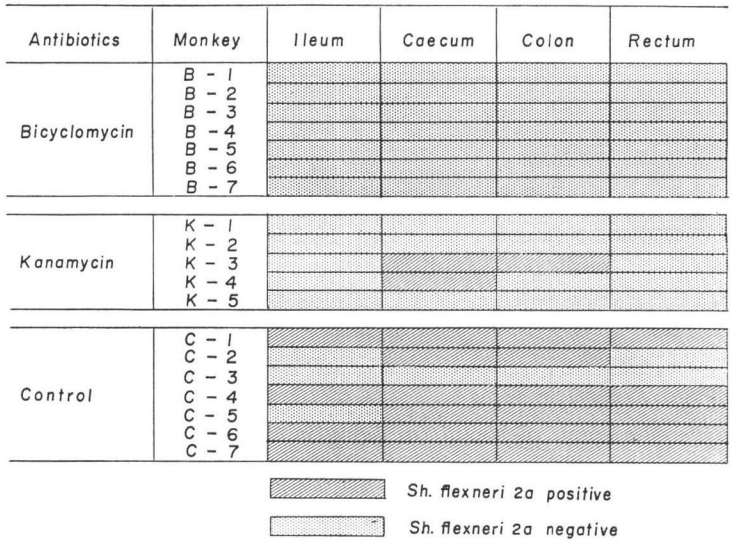


Fig. 5. Bacteriological findings in intestine of autopsied animals.



animal that recovered spontaneously.

Bicyclomycin group (7 animals): *Sh. flexneri* was not detected in any portion of the 7 animals.

Kanamycin group (5 animals): *Sh. flexneri* was detected in the cecum and rectum of a surviving animal and in the cecum of a sacrificed animal.

These findings clearly show the efficacy of bicyclomycin on gross fecal findings and shortening of periods required for elimination of *Shigella* from the intestine and feces.

Discussion

Animal shigellosis as a model of human shigellosis has been produced by challenging

Shigella to monkeys by several different routes.[6,7,8,9] The present authors introduced the bacilli by rectal infusion according to the method of TAKASAKA *et al.*, because in contrast with other methods, this procedure generally results in lethal infections in all animals and facilitates subsequent evaluation of therapeutic efficacy of drugs. In our studies bloody mucous feces spontaneously terminated in one of the seven control animals and microbiologically normal feces were restored. This is to be regarded as an experimental variation and it seems unlikely to preclude the usefulness of this method.

In general, drugs for the treatment of intestinal infections should (1) be stable and attain high excretion in the intestinal tract, (2) act selectively against causative organisms, and be inactive on normal intestinal flora, (3) cause no transfer of drug-resistance by R-factor, and (4) cause no gastrointestinal upset.

Bicyclomycin has a narrow antimicrobial spectrum with activity against *Shigella* species, *E. coli* and *Salmonella*. All strains of these organisms in this study were shown to be susceptible to bicyclomycin. When given orally bicyclomycin is excreted in the feces where this antibiotic is highly stable and present in high concentrations. In addition, bicyclomycin causes no transfer of drug-resistance by R-factor (unpublished data).

These data suggest the therapeutic efficacy of bicyclomycin in severe intestinal infections. The effect of repeated administration of bicyclomycin on the normal intestinal flora is now under investigation.

Acknowledgement

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Bibliography

1) KAMIYA, T.; S. MAENO, M. HASHIMOTO & Y. MINE: Bicyclomycin, a new antibiotic. II. Structural elucidation and acyl derivatives. J. Antibiotics 25: 576~581, 1972
2) MIYOSHI, T.; N. MIYAIRI, H. AOKI, M. KOHSAKA, H. SAKAI & H. IMANAKA: Bicyclomycin, a new antibiotic. I. Taxonomy, isolation and characterization. J. Antibiotics 25: 569~575, 1972
3) NISHIDA, M.; Y. MINE, T. MATSUBARA, S. GOTO & S. KUWAHARA: Bicyclomycin, a new antibiotic. III. *In vitro* and *in vivo* antimicrobial activity. J. Antibiotics 25: 582~593, 1972
4) NISHIDA, M.; Y. MINE, T. MATSUBARA, S. GOTO & S. KUWAHARA: Bicyclomycin, a new antibiotic. IV. Absorption, excretion and tissue distribution. J. Antibiotics 25; 594~601, 1972
5) TAKASAKA, M.; S. HONJO, F. CHO, K. IMAIZUMI & H. OGAWA: Bacillary dysentery in cynomolgus monkeys following the administration with *Shigella flexneri* 2a by the anal route. Japan. J. Med. Sci. Biol. 24: 379~385, 1971
6) TAKASAKA, M.; S. HONJO, S. IMAIZUMI & H. OGAWA: Dysentery produced by oral administration of the colonic contents of cynomolgus monkeys infected with *Shigella flexneri* 2a. Japan. J Med. Sci. Biol. 23: 249~254, 1970
7) TAKASAKA, M.; S. HONJO, S. FUJIWARA, K. IMAIZUMI, H. OGAWA, R. NAKAYA & A. NAKAMURA: Shigellosis in cynomolgus monkeys. VI. The inoculation with various doses of *Shigella flexneri* 2a into cecal lumen. Japan. J. Med. Sci. Biol. 21: 275~281, 1968
8) TAKASAKA, M.; S. HONJO, T. FUJIWARA, K. IMAIZUMI & H. OGAWA: Experimental infection with small doses of virulent *Shigella flexneri* 2a in cynomologus monkeys. Clinical and bacteriological observations. Japan. J. Med. Sci. Biol. 20: 195~198, 1967
9) TAKASAKA, M.; S. HONJO, K. IMAIZUMI, H. OGAWA, R. NAKAYA, A. NAKAMURA & K. MISE: Experimental infection with *Shigella sonnei* in cynomolgus monkeys. Japan. J. Med. Sci. Biol. 22: 389~393, 1969