



**Memorandum**

*Rec'd 8/30/02 jib*

Date: **AUG 23 2002**

From: Director, Division of Standards and Labeling Regulations, Office of Nutritional Products, Labeling and Dietary Supplements, HFS-820

Subject: 75-Day Premarket Notification of New Dietary Ingredients

To: Dockets Management Branch, HFA-305

New Dietary Ingredient: Agaro-oligosaccharide

Firm: Takaro Shuzo Co., Ltd.

Date Received by FDA: October 18, 2001

90-Day Date: January 16, 2002

In accordance with the requirements of section 413(a) of the Federal Food, Drug, and Cosmetic Act, the attached 75-day premarket notification and related correspondence for the aforementioned new dietary ingredient should be placed on public display in docket number 95S-0316 as soon possible since it is past the 90-day date. Thank you for your assistance.

*Felicia B. Satchell*  
Felicia B. Satchell

Attachments

*955-0316*

*RPT103*

 TAKARA SHUZO CO., LTD.

Biomedical Group

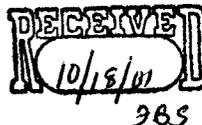
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**Takara**  
BIOMEDICALS

**COPY**  
78288



16 October, 2001

Division of Standards and Labeling Regulations  
Office of Nutritional Products, Labeling, and  
Dietary Supplements (HFS-820)  
Center for Food Safety and Applied Nutrition  
Food and Drug Administration  
200 C Street, SW  
Washington, DC20204

Re : Premarket Notification of New Dietary Ingredient "Agaro-oligosaccharide"

To Whom It May Concern :

In accordance with 21CFR Section 190.6 and Section 413 of the Federal Food, Drug, and Cosmetic Act, we hereby submit our Premarket Notification for marketing of new dietary ingredient "Agaro-oligosaccharide"



Ikunoshin Kato, Ph.D.

Executive Vice President

President, Biomedical Group

Director, Biotechnology Research Laboratories

**ORIGINAL**

**COPY**

## **Premarket Notification of New Dietary Ingredient**

# **Agaro-oligosaccharide**

**October 2001**

**TAKARA SHUZO CO.,LTD.**

Research Laboratory & Planning and Development Office  
Biotechnology Research Laboratories

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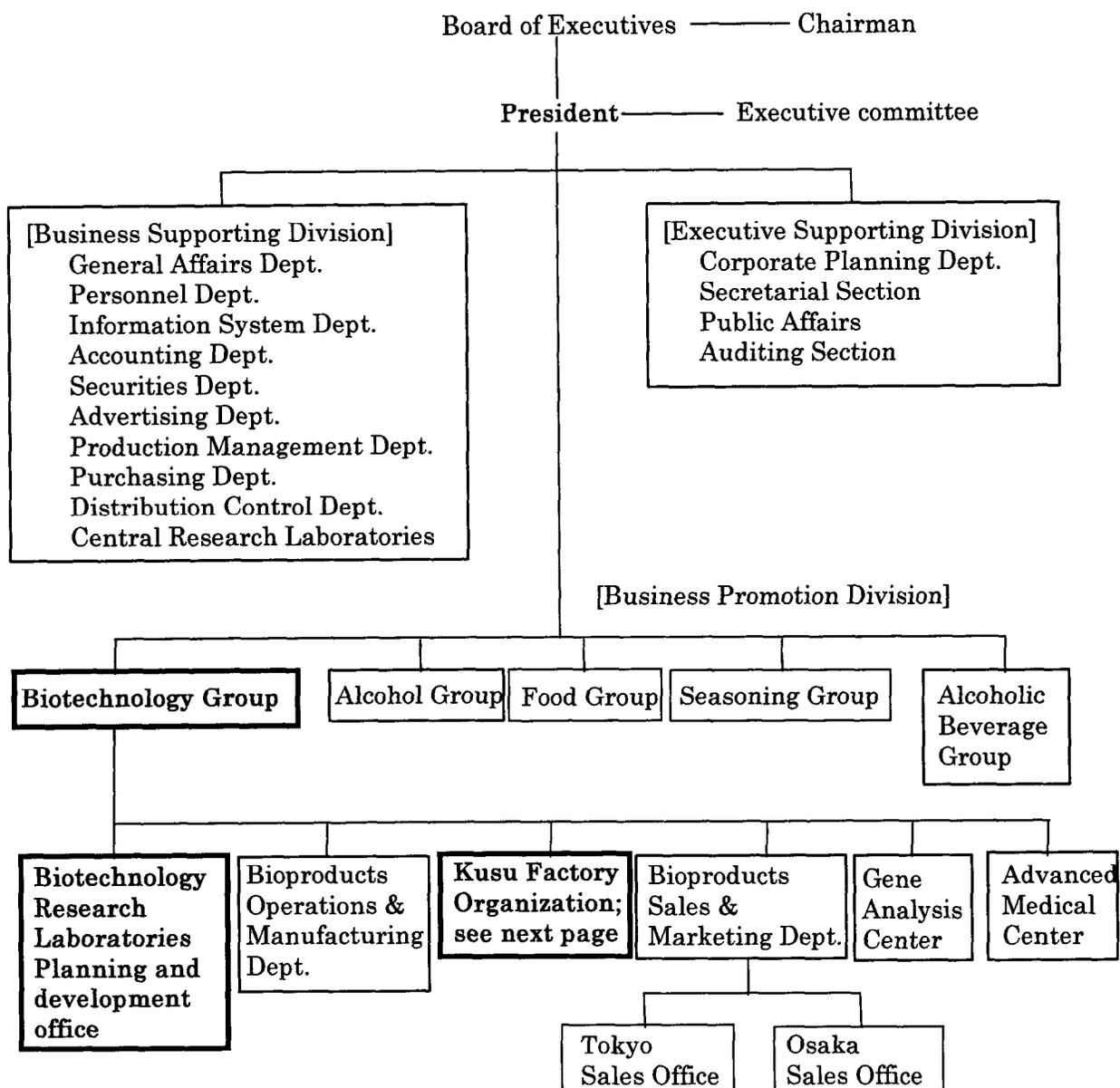
# Premarket Notification of New Dietary Ingredient "Agaro-oligosaccharide"

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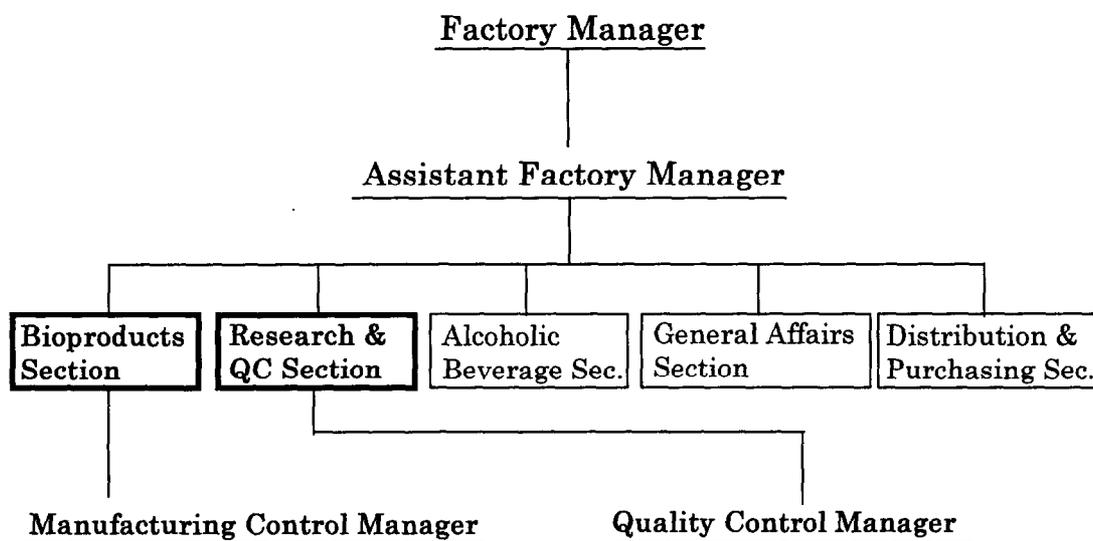
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**1. Corporate information****1.1 Name and Address of the Manufacturer****(a) Administrative Head Office;****TAKARA SHUZO CO.,LTD.****Shijo-Karasuma Higashi-iru,****Shimogyo-ku, Kyoto 600-8688, Japan****(b) Research Laboratory & Planning and Development Office****Biotechnology Research Laboratories****TAKARA SHUZO CO.,LTD.****3-4-1 Seta, Otsu,****Shiga, 520-2193, Japan****Phone:+81-77-543-7235, FAX:+81-77-543-2312****(c) Manufacturing Facility;****KUSU FACTORY****TAKARA SHUZO CO.,LTD.****1315, Minamigomizuka, Kusu-Cho****Mie-Gun, Mie, 510-0104, Japan****Phone:+81-593-97-3123 FAX:+81-593-97-3125**

## 1.2 Organization of Takara Shuzo Co.,Ltd.



## 1.3 Organization of Kusu Factory of Takara Shuzo Co.,Ltd.



**2 . Name of New Dietary Ingredient**

**“ Agaro-oligosaccharide ”**

### 3. Identity Information

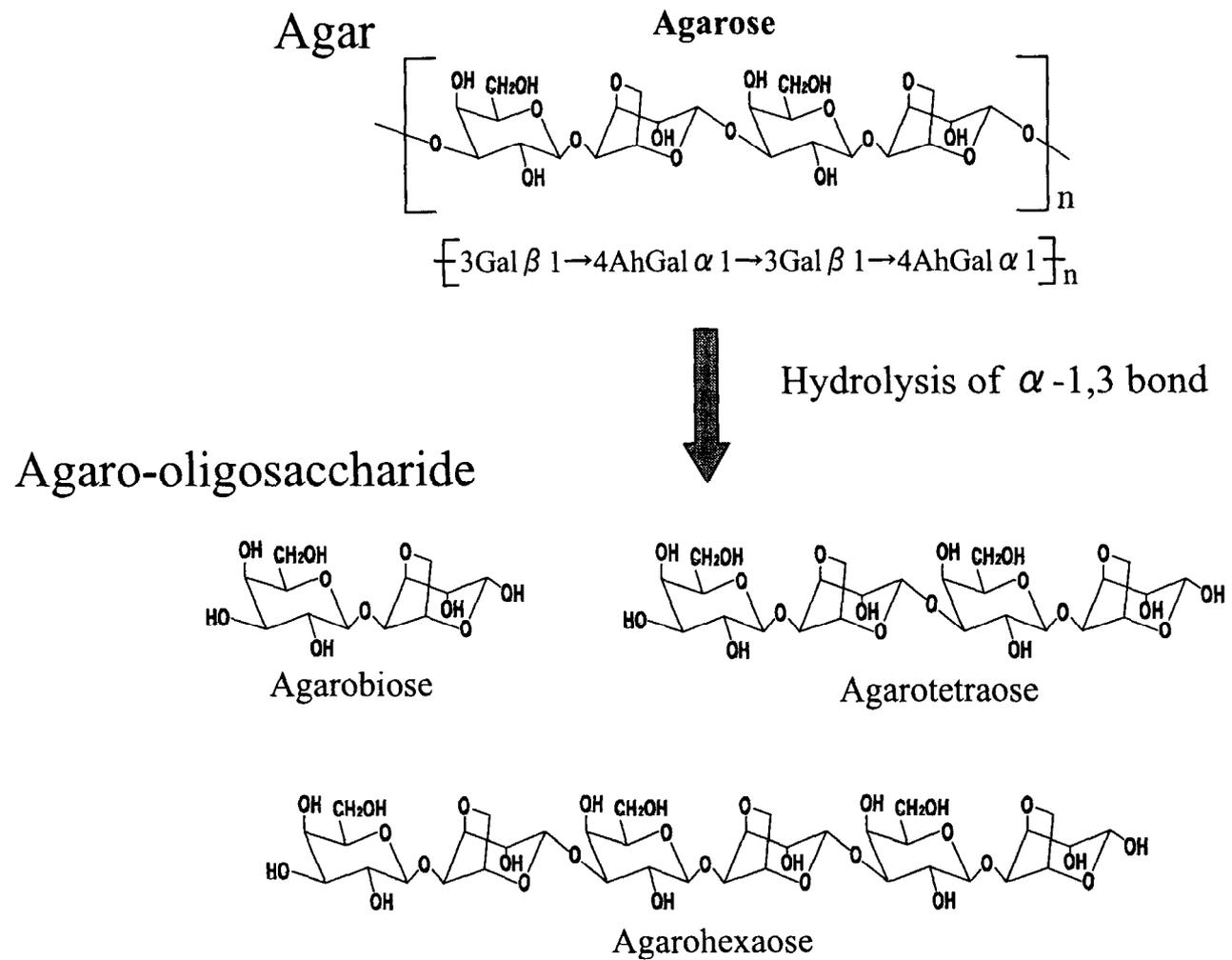
#### 3-1 What is “Agaro-oligosaccharide”?

The types of seaweed, with Japanese people enjoy to eat, include red seaweed such as Ogonori (laver, using roll-Shushi, *Gracilaria vermiculophylla*) and Tengusa (agar weed, source of agar, belong to family *Gelidiaceae*) as well as brown seaweed such as Kombu and Wakame. Agar (Kanten in Japanese) is the food richest in dietary fiber and has long been part of the Japanese diet.

Agarose is the chief component of agar (comprising about 70% of it). Agarose has a structure of a repetition of Agarobiose units, each of which is an alternately connected  $\beta$ 1,4 linked D-galactose residue and  $\alpha$ 1,3 linked 3,6-anhydro-L-galactose residue<sup>1)</sup> (Fig. 1).

It is generally known that agar does not readily solidify in the presence of mildly acidic foodstuffs such as fruit. This is because Agarose is readily broken down into fragments of smaller molecular weight by weak acids. Reacting Agarose with a weak acid such as citric acid at 95°C for about two hours yields primarily Agarobiose, Agarotetraose and Agarohexose, which are referred to as “Agaro-oligosaccharide”, because the  $\alpha$ 1,3 linkage between 3,6-anhydro-L-galactose residue and the D-galactose residue is readily hydrolyzed by the weak acid<sup>2)</sup> (Fig. 1). Under such mild reaction conditions, the  $\beta$ 1,4 linkage between the D-galactose residue and the 3,6-anhydro-L-galactose residue is not hydrolyzed. The  $\alpha$ 1,3 linkage of Agarose is selectively hydrolyzed because the reducing terminal residue of 3,6-anhydrogalactose is rich in reactivity. The reaction occurs in liquid even at standard temperature in 0.1 N hydrochloric acid, the same conditions associated with gastric juice. This means that “Agaro-oligosaccharide” is formed within the body when agar itself, such as *mitsumame* (Japanese gelatin cubes) or *tokoroten* (gelidium), is ingested. As will be more concretely stated in Section 5, Efficacy Information, the authors’ research has led to the discovery of physiological actions relating to “Agaro-oligosaccharide”, which can be easily derived from agar, such as an anti-oxidation activity by the suppression of Nitrogen monoxide (NO) (Subsection 5-1), an anti-inflammation activity by the suppression of Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (5-2), an anti-inflammation activity by the suppression of Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ), Interleukin-1  $\beta$  (IL-1 $\beta$ ), and Interleukin-6 (IL-6) (5-3), the suppression of such inflammatory cytokines production caused by Heme oxygenase(HO-1) induction (5-4), and preventive and therapeutic effects in chronic Rheumatoid arthritis models (5-5).

No such activity has been noted, however, in neo-Agaro-oligosaccharides, whose



**Fig.1 Formation of Agaro-oligosaccharides**

reducing terminal produced upon hydrolysis at the  $\beta$ 1,4 linkage is galactose.

“Agaro-oligosaccharide” is formed by hydrolyzing of the  $\alpha$ 1,3 linkage between anhydrogalactose and galactose in agar through heat and exposure to acidic conditions. “Agaro-oligosaccharide” is a mixture of even-numbered oligosaccharides, mainly with 2, 4, or 6 sugars with an anhydrogalactose at its reducing terminal.

### **3-2 Production of “Agaro-oligosaccharide”**

We use an agar powder which is manufactured from Tengusa and Ogonori, called                     

### **3-3 Physical properties of “Agaro-oligosaccharide”**

“Agaro-oligosaccharide” is a dietary ingredient with the following physical properties, all of which make it very easy to handle.

- (1) Little taste and no flavor
- (2) Low viscosity up to 20% w/w concentration
- (3) Colorless
- (4) Stable at 40°C for 80 days
- (5) Relatively heat-stable under acidic conditions

## 4. Safety Information

### 4-1 The safety of agar, the material of “Agaro-oligosaccharide” , as a food

Agar, made from a red seaweed (e.g., *Tengusa*, *Ogonori* ), is a traditional food product that traces its roots to Japan. This long-enjoyed ingredient boasts a 400-year history. It finds wide use in a variety of processed traditional foods, such as *tokoroten* (gelidium), *mitsumame* (Japanese gelatin cubes) and agar gelatin, which has many applications, Japanese sweets, and others.

In 1999, 8,240 metric tons of agar were produced worldwide. Japanese production, at 940 tons, accounted for 11% of this, and ranked third behind that in Chile and the People’s Republic of China. But Japanese agar production is unable to keep pace with consumption in the nation, so seaweed for agar production is imported from Chile, Africa and other locations. The Japanese consume over 2,000 tons of agar, which amounts to one-third of worldwide consumption.

Japanese food hygiene laws classify agar as a “natural product,” as do the laws of most other countries. The Food and Drug Administration of the United States finds agar generally recognized as safe (GRAS) when used as a food.

### 4-2 Sales history of products containing “Agaro-oligosaccharide” in Japan

A beverage containing about 60 mg of “Agaro-oligosaccharide”, called “Liquid Agar”, went on the market in July 1998 and 3.6 million bottles were sold during the three-year period ending June 2001. Consumers lodged no complaints about the drink, a fact that demonstrates the safety of “Agaro-oligosaccharide”.

### 4-3 Bacterial reverse mutation test of “Agaro-oligosaccharide”

The mutagenicity of “Agaro-oligosaccharide” was examined using histidine-requiring tester strains of TA 98, TA 100, TA 1535 and TA 1537 (*Salmonella typhimurium*) and a tryptophane-requiring strain of WP2uvrA (*Escherichia coli*) either in the presence or absence of metabolic activation systems.

The test was conducted using the amounts of 312.5, 625, 1,250, 2,500 and 5,000  $\mu\text{g}/\text{plate}$ . The number of revertant colonies in the plates treated with the test substance of the tester strains did not increase to at least twice the number in the concurrent negative controls in a dose-dependent manner. Further, no bactericidal effect or precipitation of the test substance was noted.

The findings reveal that “Agaro-oligosaccharide” had no mutagenicity under these experimental conditions (Attached document 8-1).

#### **4-4 Acute oral toxicity test of “Agaro-oligosaccharide”**

A group of ten SD rats (five males and five females) were given a single oral dose of “Agaro-oligosaccharide” at 0 (control) or 2,000 mg/kg by gavage in order to observe for signs of toxicity for 14 days and estimate LD<sub>50</sub> values (Attached document 8-2).

(1) Clinical signs and mortality

No deaths resulted, and no systemic responses were noted in any animal throughout the study.

(2) Body weight

No differences in mean body weight on days 2, 3, 5, 8, and 15 after treatment were present between the control and treatment groups.

(3) Macroscopic findings

No treatment-related change was noted.

(4) LD<sub>50</sub> values

The LD<sub>50</sub> value for male or female rats given the test substance was demonstrated to be greater than 2,000 mg/kg body weight.

#### **4-5 Chronic oral toxicity test of “Agaro-oligosaccharide”**

A group of five ICR mice was kept in a plastic cage with free access to drinking water only in the form of “Agaro-oligosaccharide” (3%) or tap water (control). Signs of toxicity, changes in body weight, and abnormalities in internal organs were checked for over a 32-week period.

(1) Clinical signs and mortality

No animal died, and no abnormalities were noted in any animal throughout the study.

(2) Body weight

There was no significant difference between the average body weights of the “Agaro-oligosaccharide” group and control group throughout the study (Fig. 2).

(3) Abnormalities in internal organs

There were no abnormalities noted in the internal organs in any animal of the “Agaro-oligosaccharide” group (Fig. 3).

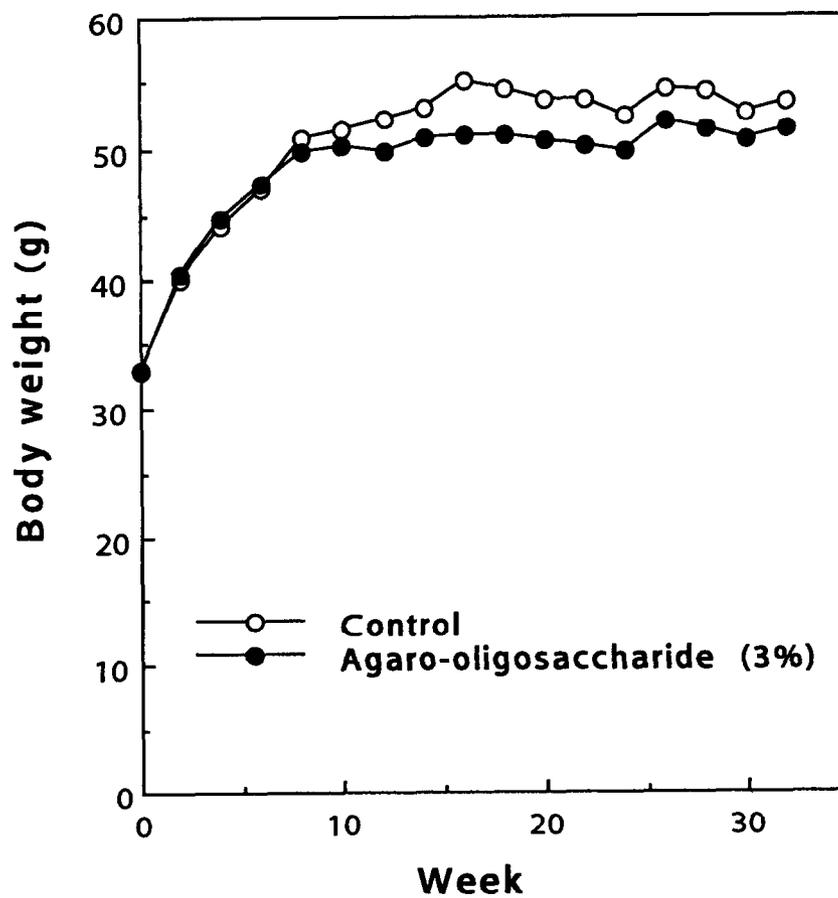
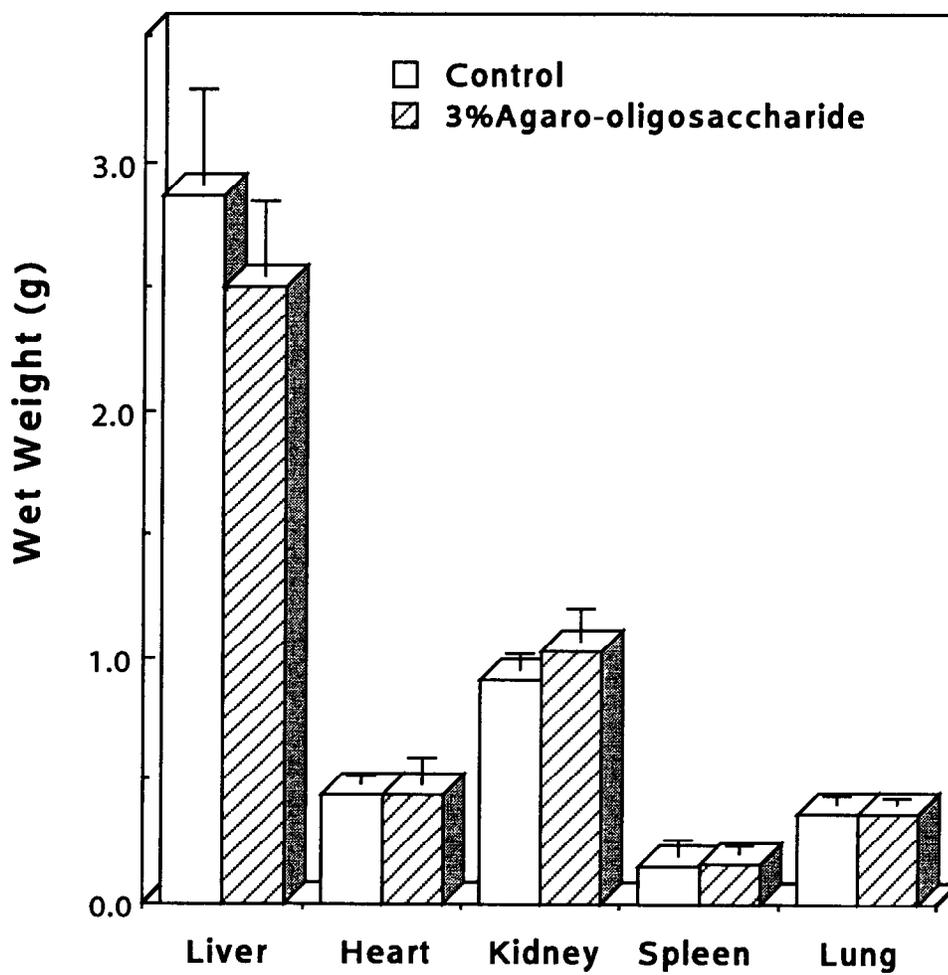


Fig.2 Variation of body weight of ICR mouse fed with or without 3% Agarose-oligosaccharides



**Fig.3 Wet Weight of internal organs of ICR mice fed with or without 3% Agar-oligosaccharide**

## 5. Efficacy Information

### 5-1 Suppression of Nitrogen monoxide (NO) production by “Agaro-oligosaccharide”

Nitrogen monoxide (NO) is a protective substance for the living body that destroys foreign objects such as pathogenic bacteria through oxidation as well as an essential information transmitter that regulates the vascular, nervous and immune systems<sup>3)</sup>. However, an excessive amount of NO produced by inducible NO synthetase (iNOS), which is induced by external factors, acts as a strongly active oxidant, to cause cell and gene damage. The treatment of mouse peritoneal macrophages with lipopolysaccharide (LPS) and Interferon- $\gamma$  (INF- $\gamma$ ), both of which are NO inducers, stimulates NO production. When Agarobiose is added at this stage, the amount of induced NO decreases in proportion to the amount of Agarobiose added. In the presence of Agarobiose, the iNOS production was investigated by western blotting after 12 hours of NO induction. The production of iNOS had been clearly suppressed<sup>4,5)</sup>. This demonstrated that “Agaro-oligosaccharide” suppresses NO production by suppressing iNOS production.

As a link between NO accumulation and Rheumatoid arthritis (RA), serum NO concentration in RA patients is known to be elevated<sup>6,7)</sup>. Although the cells that produce NO have not been clearly identified, elevated levels of NO have been located in the synovial membranes<sup>6,7)</sup>, which is thought to come from activated macrophages and neutrophils. At the sites of inflammation, a high incidence of iNOS in activated macrophages leads to the production of a large amount of NO. NO reacts with activated oxygen,  $O_2^-$ , produced by activated neutrophils at the site of inflammation, to generate the highly toxic oxidant,  $ONOO^-$ <sup>8)</sup>. In summary, the large amount of NO produced is thought to cause deterioration of RA. This suggests that the suppression of NO production by “Agaro-oligosaccharide” may work to reduce inflammations at sites of RA.

### 5-2 Suppression of Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production by Agaro-oligosaccharide

Anti-inflammatory agents have long been used in the treatment of RA. Cyclooxygenase-2 (COX-2) which produces Prostaglandin E<sub>2</sub> from arachidonic acid originating from phospholipids in cell membranes, is expressed through induction by mitogenic stimuli in the synovial tissues of RA patients<sup>9)</sup>. The PGE<sub>2</sub> produced through the reaction of COX-2 accelerates blood flow and intensifies edema and leukocyte infiltration. Anti-inflammatory agents are regularly used to inhibit the action of

COX-2, thereby suppressing PGE<sub>2</sub> production and lessening the intensity of the inflammation.

The effect of “Agaro-oligosaccharide” on the production of PGE<sub>2</sub> stimulated by LPS was investigated in the monocytes derived from human PBMC. In the human monocytes, about twice the amount of PGE<sub>2</sub> was produced during stimulation with LPS as compared to when no stimulation was provided. But the addition of “Agaro-oligosaccharide” before LPS stimulation suppressed PGE<sub>2</sub> production in a concentration dependent manner<sup>4)</sup>. It was also determined that this suppression comes not from the inhibition of COX-2 but is rather specific inhibition to the cascade in which COX-2 is involved.

“Agaro-oligosaccharide” is expected to reduce inflammations without causing adverse reactions, given that its suppression of PGE<sub>2</sub> production specifically inhibits the COX-2 cascade.

### **5-3 Suppression of Tumor Necrosis Factor- $\alpha$ (TNF- $\alpha$ ), Interleukin-1 $\beta$ (IL-1 $\beta$ ) and Interleukin-6 (IL-6) production by “Agaro-oligosaccharide”**

It is hypothesized that TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and other inflammatory cytokines are deeply involved in the conditions of RA<sup>10)</sup>. Recent research on RA therapy has focused on these inflammatory cytokines. The suppression of TNF- $\alpha$  may in turn suppress migration and infiltration of leukocytes and bring to the improvement in the condition of RA. It has also been shown that the suppression of TNF- $\alpha$  suppresses the production of IL-1  $\beta$  and IL-6<sup>11-13)</sup>.

The effect of “Agaro-oligosaccharide” on the production of inflammatory cytokines which is produced through LPS stimulation was investigated in monocytes derived from human PBMC. The LPS stimulated production of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in human monocytes were about 60, 6, and 4 times each greater than those without stimulus of LPS. The production of these inflammatory cytokines was noted in each instance. By the addition of “Agaro-oligosaccharide” before the stimulation, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 production were suppressed in a concentration dependent manner<sup>4)</sup> of “Agaro-oligosaccharide”.

The suppressive action of “Agaro-oligosaccharide” on these inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$  and IL-6) is believed to have the ability to relieve the inflammation and joint damage associated with RA. It is hoped to be useful against chronic rheumatoid arthritis.

#### **5-4 The induction of Heme oxygenase-1 (HO-1) by “Agaro-oligosaccharide” to cause the suppression of inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , and IL-6)**

Heme oxygenase is an enzyme that degrades heme into iron, CO (carbon monoxide), and biliverdin. The enzyme is known to exist in the forms of inducible HO-1 and structural HO-2 and HO-3<sup>14-16</sup>). Recent research has shown that the carbon monoxide (CO) which is produced under the action of Heme oxygenase, suppresses the production of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in macrophages stimulated by LPS, suggesting the anti-inflammatory properties of the enzyme<sup>17</sup>).

An investigation was carried out on Heme oxygenase-1 (HO-1) induction by “Agaro-oligosaccharide” in the macrophage cell line, RAW264.7. HO-1 was not occurred in cultures of the cells alone, but by the addition of “Agaro-oligosaccharide”, HO-1 was strongly induced in a concentration-dependented manner<sup>4</sup>). Given this, it is suspected that “Agaro-oligosaccharide” exhibits its anti-inflammatory properties by inducing HO-1 to generate CO and thereby suppress the production of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and other inflammatory cytokines.

#### **5-5 The preventive and therapeutic effects of “Agaro-oligosaccharide” in chronic Rheumatoid arthritis models**

An investigation was carried out on the preventive and therapeutic effects of “Agaro-oligosaccharide” on type II collagen-induced arthritis model in mice. The mice were allowed to freely drink water containing “Agaro-oligosaccharide” from the first time they were sensitized to collagen (in the investigation of preventive effects) or after immunity boosting (in the investigation of therapeutic effects). Comparing the effect on arthritis score and the incidence with those of the control group revealed a significant reduction of arthritis score in the group consuming 3% “Agaro-oligosaccharide” water as well as a significant reduction in the incidence in that group<sup>18</sup>).

This type II collagen-induced arthritis model is considered the resemble condition of human chronic Rheumatoid arthritis, because the immunity contributed to the severity and chronic nature of disease<sup>19</sup>). It is expected that “Agaro-oligosaccharide” will be useful against human chronic Rheumatoid arthritis.

#### **5-6 Conclusion**

“Agaro-oligosaccharide” has been demonstrated to bring about the following physiological actions:

- 1) Suppression of Nitrogen monoxide (NO) production
- 2) Suppression of PGE<sub>2</sub> production

- 3) Suppression of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 production
- 4) Induction of HO-1
- 5) Preventive and therapeutic effect in chronic Rheumatoid arthritis models

“Agaro-oligosaccharide” suppresses the production of PGE<sub>2</sub> and NO, key mediators in the onset of inflammation, as well as the inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6. These facts suggest that it may have anti-inflammatory properties. Its mechanism for suppressing inflammatory cytokines production is postulated to lie in its induction of HO-1. “Agaro-oligosaccharide” also exhibits preventive and therapeutic actions in *in vivo* models of chronic inflammation. It is thus concluded that “Agaro-oligosaccharide”, produced by hydrolyzing agar, is “useful as a new dietary ingredient that maintains or improves joint function.”

### Reference

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## 6. Use and application information

### 6.1 Specifications of "Agaro-oligosaccharide"

Appearance	:
pH (1% solution)	:
water content	:
Ash content	:
Arsenic	:
Heavy metals	:
Number of general bacteria	:
Coliform	:
Agaro-oligosaccharide	:

### 6.2 How to use "Agaro-oligosaccharide"

We recommend to take a product (Drink or Granules) containing 100-500mg "Agaro-oligosaccharide" in a day.

### 6.3 Efficacy of "Agaro-oligosaccharide"

"Agaro-oligosaccharide" possesses the ability to suppress the excess production of Nitrogen monoxide, Prostaglandin  $E_2$  production and inflammatory cytokines production such as Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ), Interleukin-1  $\beta$  (IL-1  $\beta$ ) and Interleukin-6 (IL-6).

It is concluded that Agaro-oligosaccharide, produced by hydrolyzing agar, is useful as a new dietary ingredient that maintains or improves joint function.

## 7. Labeling information

Products by use of "Agaro-oligosaccharide"

### Health Drink "**Kurozu Kanten**"

Net content : 50ml in brown bottle

Packaging : 30 bottles per carton

(One carton holds three cardboard boxes containing 10 bottles)

#### < Ingredients >

#### < Nutrition Facts >

Values in one serving size 1 bottle (50ml)

Energy	:	9	kilo calories
Protein	:	0	g
Lipids	:	0	g
Saccharides	:	2.3	g
Sodium	:	16	mg

#### < Description of the product >

This product is a drink containing 200mg of "Agaro-oligosaccharide" and 1.25ml of the traditional vinegar *Sakamoto-no-Kurozu*. The drink is low in calories (a single 50ml bottle contains only 9 kilocalories) and sugar.

Agaro-oligosaccharide possesses the ability to suppress the excess production of Nitrogen monoxide, Prostaglandin E<sub>2</sub> production and inflammatory cytokines production such as Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ), Interleukin-1  $\beta$  (IL-1  $\beta$ ) and Interleukin-6(IL-6).

#### < Instruction >

- We recommend to take two bottles a day .

#### < Caution >

- It might cause loose bowels in case too much drink .

#### < Preservation >

- It should not keep in direct sunshine, at a high temperature and frozen temperature.
- It is not a problem to be turbid or precipitated during preservation.

Final Report

Title : Bacterial reverse mutation test of Agaro Oligosaccharide

PROJECT No. H-00266

Nippon Experimental Medical Research Institute Co., Ltd

3303-58 Ohaza Ohdo, Agatsuma machi, Agatsuma gun, Gunma-ken.

Date of reporting: December 15, 2000.

## STATEMENT OF COMPLIANCE

Title : Bacterial reverse mutation test of Agaro Oligosaccharide

PROJECT No. H-00266

I, the undersigned, hereby declare that this report is the final English version of the original report that has written in Japanese language. Further, I declare that there is no adverse affect on the quality or integrity of the study or the interpretation of the results due to translation of the report.

M. Kashima

Date: May 8, 2001

Masaaki Kashima, D.V.M.

Managing Director

Nippon Experimental Medical Research Institute Co., Ltd.

## STATEMENT OF COMPLIANCE

Title : Bacterial reverse mutation test of Agaro Oligosaccharide

PROJECT No. H-00266

I, the undersigned, hereby declare that this report is the English version of the original report that has written in Japanese language. Further, I declare that the data are exactly reflected in this report and similar to that of the original (Japanese) report.



Golam Sarwar, Ph.D.

Study Director (translator)

Nippon Experimental Medical Research Institute Co., Ltd.

Date: May 8, 2001

## PREPARATION OF THE FINAL REPORT

Title : Bacterial reverse mutation test of Agaro Oligosaccharide

PROJECT No. H-00266

In this study, the Ordinance that describing the Standard for Pre-clinical Safety Studies on Drugs, Ministry of Health and Welfare, Japan (Ministry of Health and Welfare Ordinance No. 21: March 26, 1997) was followed as reference.

Further, this study has been conducted in accordance with the methods describing in this report, and the data are accurately reflected in this report.

Date: December 15, 2000.

Golam Sarwar, Ph. D. (Impression of the seal)

Study director

Nippon Experimental Medical Research Institute Co., Ltd.

Title : Bacterial reverse mutation test of Agaro Oligosaccharide  
PROJECT No. H-00266

1. Purpose

Bacterial reverse mutation test was conducted to clarify whether Agaro Oligosaccharide had mutagenic potential or not.

2. Compliance with GLP

In this study, the Ordinance that describing the Standard for Pre-clinical Safety Studies on Drugs, Ministry of Health and Welfare, Japan (Ministry of Health and Welfare Ordinance No. 21: March 26, 1997) was followed as reference.

3. Compliance with Guidelines

The present study was conducted in compliance with the Guideline that describing the genotoxicity tests of drugs (Ministry of Health and Welfare Ordinance No. 1604: November 11, 1999) was followed.

4. Sponsor

Name : Takara Shuzo Co., Ltd.  
Biomedical Group  
Address : Seta 3-4-1, Otsu, Shiga Ken, Japan

5. Contract Laboratory

Name : Nippon Experimental Medical Research Institute Co., Ltd.  
Address : 3303-58, Ohaza Ohdo, Agatsuma machi, Agatsuma gun,  
Gunma Prefecture, Japan.

6. Testing Facility

Name : Haruna Laboratory  
Nippon Experimental Medical Research Institute Co., Ltd.  
Address : 3303-58, Ohaza Ohdo, Agatsuma machi, Agatsuma gun,  
Gunma Prefecture, Japan.  
Managing Director: Masaaki Kashima, D.V.M.

## 7. Storage of records and data

### (1) Storing period

Stored for a period that described in the Drugs Act, Ministry of Health and Welfare, Japan, Ordinance No. 26-2-3, 26-5-3 and 26-12.

### (2) Storing materials and place

① Protocol, records concern to the study, final report in original and raw-data are to be stored in the raw-data archive of Haruna Laboratory, Nippon Experimental Medical Research Institute Co., Ltd.

② Sample is to be stored in the material storage (No. 1) of the same facility.

③ Records are to be stored in the storage room of the same facility.

## 8. Schedule of the study

Initiation of the Project	:	October 18, 2000
Initiation of experiment	:	October 31, 2000
Completion of experiment:		November 13, 2000
Draft report	:	November 22, 2000
Final report	:	December 15, 2000
Completion of the Project	:	December 15, 2000

## 9. Study personnel and work responsibility

Study director, protocol preparation, test substance control, supervising, management and preparation of final reporting

: Golam Sarwar

Division of Mutation Research, Nippon

Experimental Medical Research Institute Co., Ltd

Test substance preparation : Haruki Inoue

Media & reagents

preparation and experiment : Haruki Inoue, Mutsumi Takano, Kazuko Iiduka

Colony counting : Mutsumi Takano

Data processing and judgement: Golam Sarwar, Haruki Inoue, Mutsumi Takano,

Kazuko Iiduka

4/4

Unpredicted Situation Effecting The Reliability Of The Study And Deviations From  
The Protocol

Title : Bacterial reverse mutation test of Agaro Oligosaccharide  
PROJECT No. H-00266

There were no unpredicted situation affecting the reliability of the study and no  
deviations from the protocol.

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## I. Summary

The mutagenicity of Agaro Oligosaccharide was examined using histidine requiring tester strains of TA 98, TA 100, TA 1535 and TA 1537 (*Salmonella typhimurium*) and tryptophane requiring strain of WP2uvrA (*Escherichia coli*) either in the presence or absence of metabolic activation systems.

The test was conducted at the doses of 312.5, 625, 1250, 2500 and 5000  $\mu$ g/plate. As a result, the numbers of revertant colonies in the test substance treated plates of the tester strains were not increased at least twice the concurrent negative controls in a dose-dependent manner. Further, bactericidal effect and precipitations of the test substance were not noted.

The findings concluded that Agaro Oligosaccharide had no mutagenicity under this experimental condition.

## II. Purpose of Study

Bacterial reverse mutation test was conducted to clarify whether Agaro Oligosaccharide had mutagenic potential or not.

## III. Materials and Methods

### 1. Test substance

- (1) Test substance name : Agaro Oligosaccharide
- (2) Lot No. : AG-0023-SD
- (3) Purity : 95%
- (4) Molecular weight : 1000 in average
- (5) Stability : Stable in refrigerator
- (6) Name of impurities : Water
- (7) Appearance at ordinary temperature : White powder
- (8) Solubility : A 30% is soluble in water at 20°C and stable
- (9) Storing condition : Under refrigeration
- (10) Producing date : May 25, 2000
- (11) Validity period : 3 years from the date of production (2003. 5. 24)
- (12) Supplier
  - Name : Takara Shuzo Co., Ltd.
  - Biomedical Group
  - Address : Seta 3-4-1, Otsu, Shiga Ken, Japan
- (13) Disposal : After completion of the study, the test substance is to be forwarded to the sponsor being preserved a little portion.

### 2. Controls

#### (1) Negative control

Pure water (medicinal grade, Lot No. 99H27A, Fuso Pharmaceutical Industries Ltd) was used.

#### (2) Positive control

The substances of 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2, Lot No.

PAE1151, purity:98.7%), Sodium azide (SA, Lot No. ACK5372, purity:91.5%) and 2-Aminoanthracene (2-AA, Lot No. DSJ3206, purity:96.5%) of Wako Pure Chemical Industries Ltd and 9-Aminoacridine (9-AA, Lot No. 106F06681, purity:98.0%) of Sigma Chemical Co., Ltd were used

### 3. Preparation of test substance

#### (1) The solvent and the reason of it's selection

According to the sponsor, 30% of the test substance was found soluble and stable in water. Based on solubility and stability, pure water was used as solvent in the test.

#### (2) Preparation of test solution

To prepare the highest dosing mixture, 264 mg of test substance was weight and match up in 5 mL of pure water (medicinal grade, Lot No. 99H27A, Fuso Pharmaceutical Industries Ltd). The lower doses were prepared by serial dilution with the solvent. They were prepared just before use.

In soluble condition, the test substance was stable as temperature formation, foaming and coloration were not detected when checked macroscopically. Further, homogeneity was confirmed macroscopically.

### 4. Preparation of control substance

#### (1) Negative control

The solvent that used for the preparation of test substance was used as negative control.

#### (2) Positive control

The substances of AF-2, 9-AA and 2-AA were dissolved separately in DMSO (special grade, Lot No. ELP3545, Wako Pure Chemical Industries Ltd) and SA was dissolved in pure water (medicinal grade, Lot No. 98F22A, Fuso Pharmaceutical Industries Ltd) to prepare the required concentrations, distributed, stored at below -80°C in a deep freezer and used after thawing.

### 5. Tester strains

#### (1) Procurement

The tester strains of TA 98, TA 100, TA1535 and TA 1537 of Salmonella

typhimurium and WP2uvrA of *Escherichia coli* were obtained from Japan Bioassay Laboratory (2445 Hirasawa, Hadano shi, Kanagawa Ken). Upon receipt, they were subcultured for 8 hr, in each culture DMSO was added (8:0.7), distributed and stored at below  $-80^{\circ}\text{C}$  in a deep freezer until use. The stock cultures that confirmed for genotypes were used.

## (2) Subculturing

A 24  $\mu\text{L}$  of frozen stock (being thawed) was transferred aseptically to 12 mL of freshly prepared Nutrient broth No. 2 (Lot No 028 59355, Oxoid) and subcultured at  $37^{\circ}\text{C}$  with agitation (80 times/min) for 10 hr in a water bath shaker (L-10, Taitec Ltd) with a program unit (PU-9, Taitec). Then, optical density (OD) of each culture was measured at 660 nm using a spectrophotometer (Fuji Kogyo Co., Ltd), confirmed the cell density and used.

## 6. Media

### (1) Nutrient broth (NB)

A 2.5% solution of NB No. 2 (Oxoid) was prepared in pure water, pipetted into L-shape test tube ( $15 \times 80 \times 180$  mm) at a volume of 12 mL per tube and autoclaved.

### (2) Minimal glucose agar plates

They were purchased commercially as Vital medium AMT-0 (Lot No. DZA19E01) from Kyokuto Pharmaceutical Co., Ltd and used.

### (3) Identification of plates

Each of the plate was identified by writing the serial number in the top of the lid and the lower part.

### (4) Top agar

Top agar that used for *Salmonella* strains was prepared by mixing pre-autoclaved soft agar (consisting of 0.6% Bacto agar, Lot No. 139900XA, Difco Laboratories and 0.5% NaCl) with a solution of 0.5mM L-histidine (Lot No. 11H0197, Sigma Chemical Co. Ltd )-0.5mM D-biotin (120H0305, Sigma Chemical Co. Ltd ) at a ratio of 10:1. Similarly, soft agar was mixed with 0.5 mM L-tryptophane (Lot No. DSG 2309, Wako Pure Chemical Industries Ltd) for *Escherichia coli*.

## 7. Rat liver homogenate (S9) and S9 mix

### (1) Source of S9

The S9 (Lot No. RAA-433) of Sprague-Dawley male rats that induced by Phenobarbital and 5,6-Benzoflavone was purchased from Kikkoman Corporation, Japan, stored at below  $-80^{\circ}\text{C}$  in a deep freezer and used after thawing.

### (2) Preparation of S9 mix

The mixture of each mL consisting of 0.1 mL S9,  $4\ \mu\text{mol}$  NADPH (Lot No.050006),  $4\ \mu\text{mol}$  NADH (Lot No. 010034),  $5\ \mu\text{mol}$  glucose-6-phosphate (Lot No. 115001) of Oriental Yeast Co., Ltd.,  $33\ \mu\text{mol}$  KCl,  $8\ \mu\text{mol}$   $\text{MgCl}_2$ ,  $100\ \mu\text{mol}$  sodium phosphate buffer (pH 7.4) and adjusted to 1 mL by adding pure water. The mixture was prepared prior to use and kept in ice-cold water bath during use.

## 8. Doses

The doses of 5, 10, 50, 100, 500, 1000 and  $5000\ \mu\text{g/plate}$  were used in dose determination test. Whereas the doses of 312.5, 625, 1250, 2500 and  $5000\ \mu\text{g/plate}$  were used in mutagenicity test.

## 9. Experimental procedure

The dose determination and mutagenicity tests were carried out in pre-incubation method<sup>1,2)</sup> either in the presence or absence of metabolic activation system.

## 10. Experimental condition

### (1) Treatment

A 0.5 mL of S9 mix or phosphate buffer(pH 7.4), 0.1 mL of each concentration of test substance or negative or positive control, 0.1 mL of each tester strain were taken into heat sterilized glass tube ( $13 \times 100\ \text{mm}$ ), mixed and agitated for 20 min at  $37^{\circ}\text{C}$  in water bath shaker (Iwaki Glass). After that, 2 ml of molten top agar of  $45^{\circ}\text{C}$  was taken into each tube, mixed and plated by pouring into pre-numbered minimal glucose agar plate. Duplicate plates were used for each dose in dose determination and mutagenicity tests except for the negative control of mutagenicity test in which triplicate plates were used.

(2) Used amount of positive control in each tester strain

Tester strains	Without metabolic activation (-S9)		With metabolic activation (+S9)	
	Substances	Dose( $\mu$ g/plate)	Substances	Dose( $\mu$ g/plate)
Salmonella				
Typhimurium				
TA 98	AF-2	0.1	2-AA	0.5
TA 100	AF-2	0.01	2-AA	1.0
TA 1535	SA	0.5	2-AA	2.0
TA 1537	9-AA	80.0	2-AA	2.0
Escherichia coli				
WP2uvrA	AF-2	0.01	2-AA	10.0

(3) Sterility test

The test substance of highest dose and S9 mix were plated for sterility test.

(4) Incubation

After solidification of top agar, plates were inverted and incubated at 37°C for 48 hr in an incubator (MFR-116S, Isuzu Industries Ltd).

11. Colony count and judgment

(1) Colony count

After completion of incubation, the colonies of all plates except for positive control plates were counted manually. Each of the positive control plate was counted three times (by rotating at an angle of 120°) using an auto colony counter (Olympus OL-502A, Yoshikawa Industries Ltd) and average of such counts was expressed as revertant colonies per plate. The mean number of colonies in duplicate or triplicate plates was expressed as revertant colonies per dose.

(2) Observation of background

During colony count, bacterial growth inhibition (bactericidal effect) of the test substance was determined from the background lawn under stereozoom microscope (CSZ, Uchida-Yoko Ltd) and at the same time precipitates of the test substance was also determined.

### (3) Judgment of the result

The test substance was judged positive (+) when the number of revertant colonies in the test substance treated plates increased dose dependently and became 2-fold compared to that of the negative control and this effect was reasonably reproducible and the others were judged negative (-). Bacterial growth inhibition (bactericidal effect) was judged when the lawn of a test plate was sparse or thin compared to that of the negative control plate.

### 12. Statistical evaluation

The statistical analysis was not done for the judgment.

#### IV. Results

A dose selection test was conducted at the doses of 5, 10, 50, 100, 500, 1000 and 5000  $\mu$  g/plate either in the presence or absence of metabolic activation systems to find out a dose at which the test substance inhibited bacterial growth and caused precipitations. In the test, bacterial growth inhibition (bactericidal effect) and precipitations of the test substance were not noted (Appendix 1).

Due to such results, a total of 5 doses considering 5000  $\mu$  g/plate as highest and four more lower doses of 2500, 1250, 625 and 312.5 at a nominal ration of 2 were selected and used in the mutagenicity test in either systems.

As a result, the number of revertant colonies in the test substance treated plates of all tester strains were not increased dose dependently and not became 2-fold compared to that of the negative control of each tester strain. Further, bactericidal effect and precipitations of the test substance were not noted (Fig 1&2, Appendix 2).

The positive controls of all tester strains showed marked increase in the number of revertant colonies compared to that of the corresponding negative control of each tester strain.

## V. Discussion and Conclusion

The mutagenicity of Agaro Oligosaccharide was examined either in the presence or absence of metabolic activation systems using the tester strains of TA 98, TA 100, TA 1535, TA 1537 of *Salmonella typhimurium* and the strain of WP2uvrA of *Escherichia coli*.

At first, a dose selection test was conducted at the doses of 5~5000  $\mu$ g/plate. As a result of the test, bactericidal effect and precipitations of the test substance were not noted. Due to such findings, a dose of 5000  $\mu$ g/plate as highest and four more lower doses at a nominal ratio of 2 (total: 5 doses) were selected and used in the mutagenicity test.

In the test, the numbers of revertant colonies in the test substance treated plates of all tester strains were not increased dose dependently and not became 2-fold compared to that of the negative control of each tester strain. Further, bactericidal effect and precipitations of the test substance were not noted.

The numbers of revertant colonies in the negative and positive controls of all tester strains were within mean  $\pm$  2SD of the background data (Attached sheet 1) which demonstrated that the study was conducted appropriately.

Contamination was not noted in the sterility tests that conducted during dose selection and mutagenicity tests.

According to the findings of this experimental condition Agaro Oligosaccharide was concluded as non-mutagen.

## VI. References

- 1) Maron, D.M., and Ames B.N. : Revised methods for the Salmonella mutagenicity test, *Mutation Res.*, 113, 173 - 215, 1983
- 2) Yahagi, T., Nagano, M., Seino, Y., Matsushima, T., and Okada, M. : Mutagenicities of N-nitosamines on Salmonella, *Mutation Res.*, 48, 121 – 130, 1977.

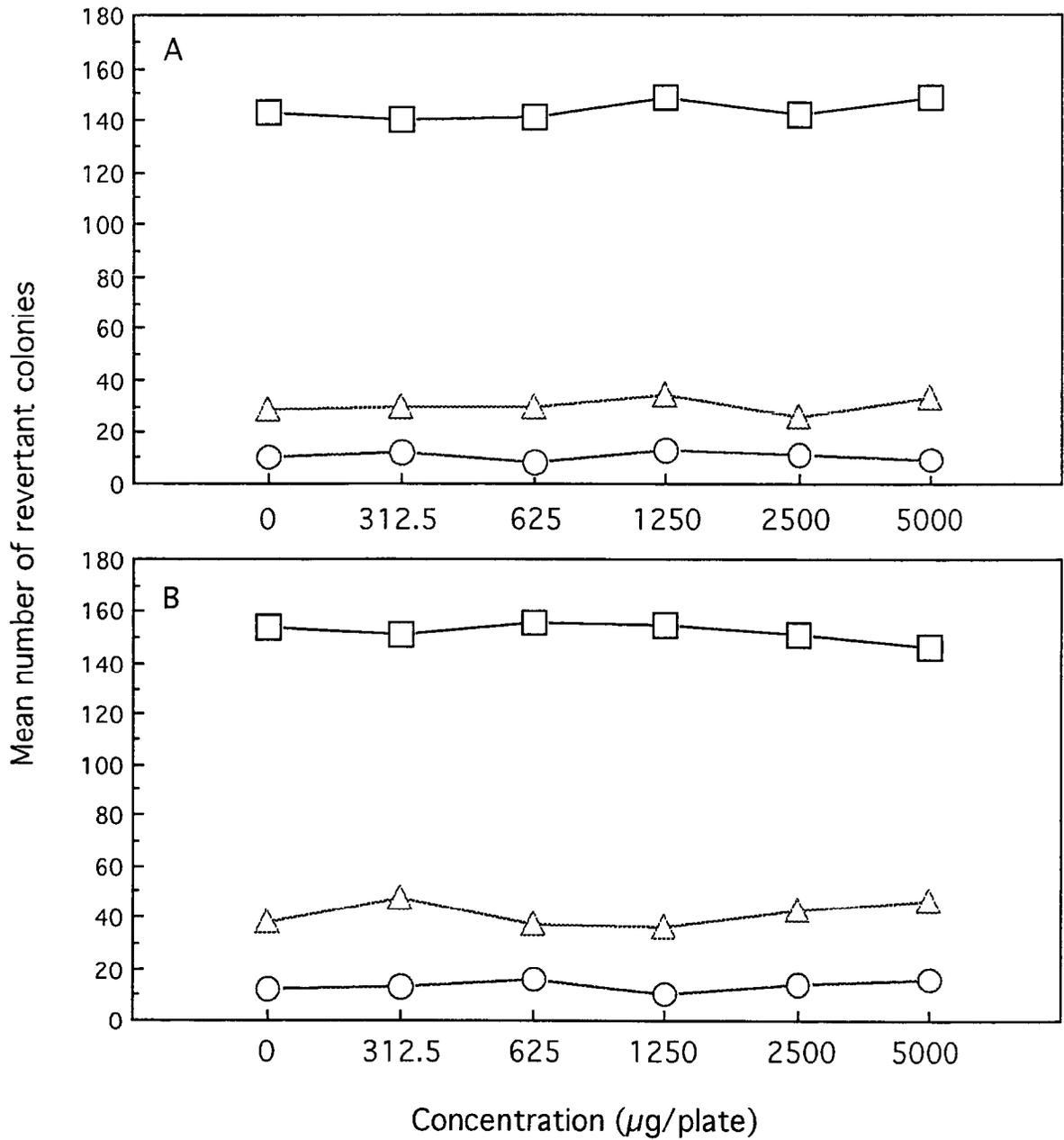


Fig. 1. Dose depending curve of Agaro Oligosaccharide (Mutagenicity test)

A : Without metabolic activation system (-S9)

B : With metabolic activation system (+S9)

□ : TA100; ○ : TA1535; △ : WP2uvrA

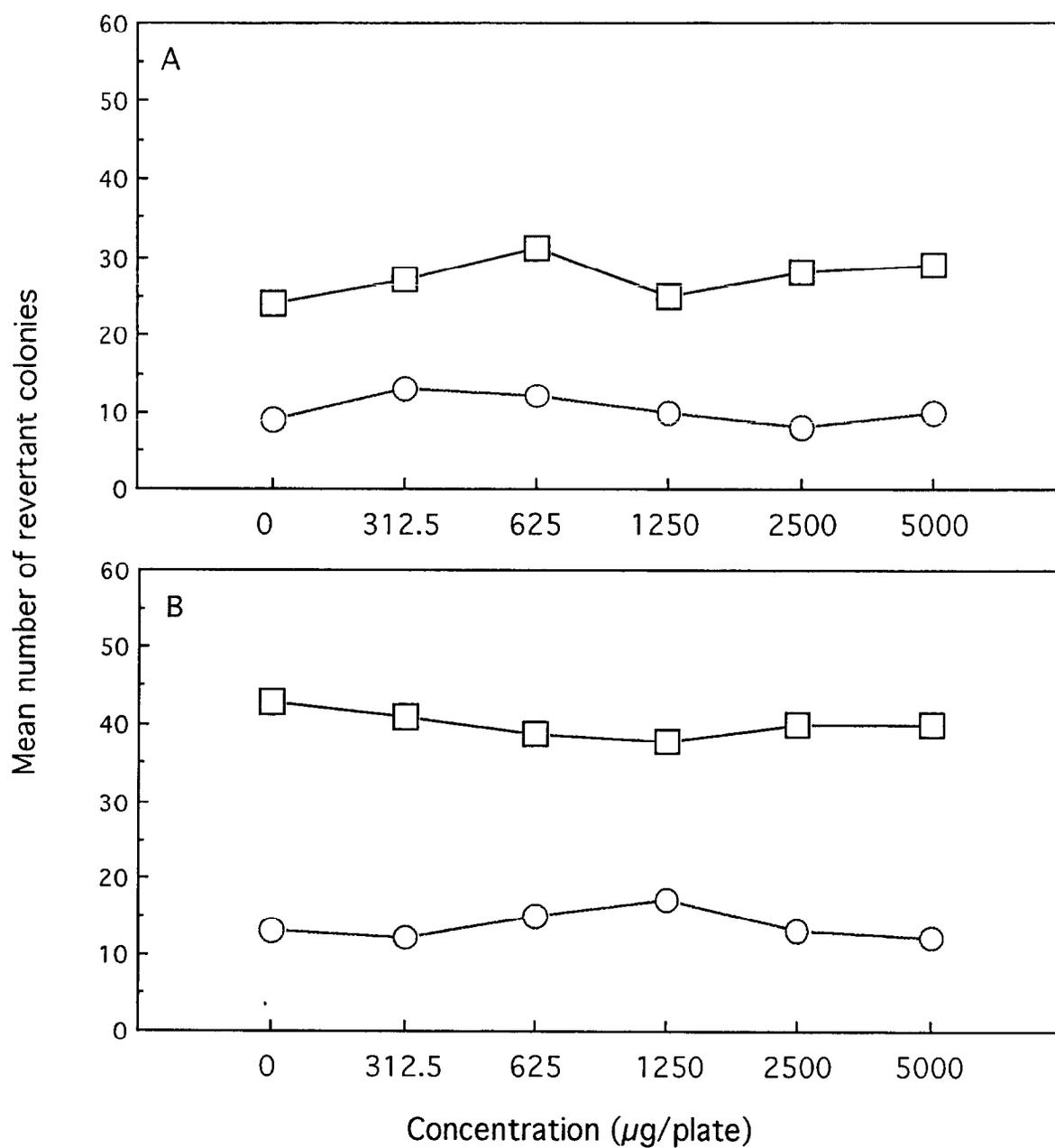


Fig. 2. Dose depending curve of Agarose Oligosaccharide (Mutagenicity test)

A : Without metabolic activation system (-S9)

B : With metabolic activation system (+S9)

□ : TA98 ; ○ : TA1537

Appendix 1. Reverse mutation test of Agarose Oligosaccharide in *S. typhimurium* and *E. coli* (Dose determination test)

With (+) or Without (-) S9 mix	Test substance concentration ( $\mu\text{g}/\text{plate}$ )	Number of revertants (number of colonies/plate) <sup>a)</sup>				
		Base-pair substitution type			Frameshift type	
		TA 100	TA 1535	WP 2 u v r A	TA 98	TA 1537
S9 mix ( - )	Solvent control	146 137 ( 142 )	9 12 ( 11 )	34 27 ( 31 )	21 26 ( 24 )	9 5 ( 7 )
	5	131 145 ( 138 )	9 7 ( 8 )	24 23 ( 24 )	28 27 ( 28 )	4 3 ( 4 )
	10	131 129 ( 130 )	6 7 ( 7 )	30 29 ( 30 )	21 19 ( 20 )	6 9 ( 8 )
	50	129 130 ( 130 )	7 8 ( 8 )	25 29 ( 27 )	15 20 ( 18 )	5 5 ( 5 )
	100	117 133 ( 125 )	14 8 ( 11 )	23 24 ( 24 )	16 25 ( 21 )	7 3 ( 5 )
	500	128 133 ( 131 )	12 7 ( 10 )	25 28 ( 27 )	17 18 ( 18 )	6 5 ( 6 )
	1000	126 128 ( 127 )	9 9 ( 9 )	33 33 ( 33 )	17 21 ( 19 )	10 8 ( 9 )
	5000	127 143 ( 135 )	6 12 ( 9 )	33 37 ( 35 )	26 21 ( 24 )	4 7 ( 6 )
S9 mix ( + )	Solvent control	152 149 ( 151 )	13 15 ( 14 )	41 37 ( 39 )	32 39 ( 36 )	15 10 ( 13 )
	5	147 145 ( 146 )	18 14 ( 16 )	35 37 ( 36 )	27 40 ( 34 )	16 10 ( 13 )
	10	151 144 ( 148 )	15 12 ( 14 )	39 35 ( 37 )	25 37 ( 31 )	17 11 ( 14 )
	50	140 143 ( 142 )	16 10 ( 13 )	31 28 ( 30 )	29 32 ( 31 )	9 10 ( 10 )
	100	157 138 ( 148 )	11 15 ( 13 )	36 32 ( 34 )	44 40 ( 42 )	11 18 ( 15 )
	500	153 150 ( 152 )	14 13 ( 14 )	36 31 ( 34 )	35 31 ( 33 )	7 9 ( 8 )
	1000	138 150 ( 144 )	17 12 ( 15 )	34 49 ( 42 )	23 27 ( 25 )	5 9 ( 7 )
	5000	132 152 ( 142 )	11 12 ( 12 )	45 40 ( 43 )	36 30 ( 33 )	8 10 ( 9 )
Positive control not requiring S9 mix	Name	A F - 2	S A	A F - 2	A F - 2	9 - A A
	Concentration ( $\mu\text{g}/\text{plate}$ )	0.01	0.5	0.01	0.1	80
	Number of colonies/plate	564 551 ( 558 )	507 484 ( 496 )	187 197 ( 192 )	554 472 ( 513 )	653 638 ( 646 )
Positive control requiring S9 mix	Name	2 - A A	2 - A A	2 - A A	2 - A A	2 - A A
	Concentration ( $\mu\text{g}/\text{plate}$ )	1	2	10	0.5	2
	Number of colonies/plate	933 961 ( 947 )	217 233 ( 225 )	1055 1001 ( 1028 )	618 579 ( 599 )	219 228 ( 224 )

<sup>a)</sup> : The average number of colonies in each concentration.

Solvent : Distilled water for injection.

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Appendix 2. Reverse mutation test of Agar Oligosaccharide in *S. typhimurium* and *E. coli* (Mutagenicity test)

With (+) or Without (-) S9mix	Test substance concentration ( $\mu\text{g}/\text{plate}$ )	Number of revertants (number of colonies/plate) <sup>a)</sup>				
		Base-pair substitution type			Frameshift type	
		TA 100	TA 1535	WP 2 u v r A	TA 98	TA 1537
S 9 m i x  ( - )	Solvent control	134	9	23	27	10
		150 ( 143 )	9 ( 10 )	29 ( 29 )	18 ( 24 )	7 ( 9 )
		144	12	34	28	9
	312.5	133	12	23	27	13
		147 ( 140 )	11 ( 12 )	37 ( 30 )	26 ( 27 )	12 ( 13 )
		128	7	31	27	12
625	154 ( 141 )	8 ( 8 )	28 ( 30 )	34 ( 31 )	11 ( 12 )	
	150	10	41	20	9	
	146 ( 148 )	15 ( 13 )	26 ( 34 )	30 ( 25 )	10 ( 10 )	
1250	134	10	26	30	5	
	149 ( 142 )	12 ( 11 )	26 ( 26 )	26 ( 28 )	11 ( 8 )	
	151	9	35	26	10	
2500	145 ( 148 )	9 ( 9 )	30 ( 33 )	32 ( 29 )	9 ( 10 )	
	155	8	32	43	13	
	155 ( 154 )	13 ( 12 )	43 ( 38 )	44 ( 43 )	12 ( 13 )	
S 9 m i x  ( + )	Solvent control	153	15	40	42	14
		158	12	49	43	11
		143 ( 151 )	14 ( 13 )	46 ( 48 )	38 ( 41 )	13 ( 12 )
	312.5	157	14	39	42	17
		155 ( 156 )	17 ( 16 )	34 ( 37 )	35 ( 39 )	12 ( 15 )
		156	12	33	40	15
625	153 ( 155 )	8 ( 10 )	38 ( 36 )	36 ( 38 )	18 ( 17 )	
	152	12	44	44	10	
	150 ( 151 )	15 ( 14 )	41 ( 43 )	35 ( 40 )	16 ( 13 )	
1250	131	12	46	37	13	
	160 ( 146 )	19 ( 16 )	47 ( 47 )	42 ( 40 )	11 ( 12 )	
	160 ( 146 )	19 ( 16 )	47 ( 47 )	42 ( 40 )	11 ( 12 )	
Positive control not requiring S9 mix	Name	A F - 2	S A	A F - 2	A F - 2	9 - A A
	Concentration ( $\mu\text{g}/\text{plate}$ )	0.01	0.5	0.01	0.1	80
	Number of colonies/plate	511	480	168	540	607
		556 ( 534 )	453 ( 467 )	181 ( 175 )	524 ( 532 )	560 ( 584 )
Positive control requiring S9 mix	Name	2 - A A	2 - A A	2 - A A	2 - A A	2 - A A
	Concentration ( $\mu\text{g}/\text{plate}$ )	1	2	10	0.5	2
	Number of colonies/plate	1021	217	837	483	239
		965 ( 993 )	163 ( 190 )	1067 ( 952 )	490 ( 487 )	274 ( 257 )

<sup>a)</sup> : The average number of colonies in each concentration.

Solvent : Distilled water for injection.

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Attached sheet 1

Historical background data (Preincubation method)

Negative control	Distilled water for injection									
	-					+				
S 9 mix										
Tester strain	TA100	TA1535	WP2uvrA	TA98	TA1537	TA100	TA1535	WP2uvrA	TA98	TA1537
N	33	29	29	29	29	33	33	29	29	29
Mean	136	11	35	24	9	142	14	42	34	12
S.D.	9	2	6	4	3	8	3	6	5	3
2S.D.	18	4	12	8	6	16	6	12	10	6
Mean-2S.D.	118	7	23	16	3	126	8	30	24	6
Mean+2S.D.	154	15	47	32	15	158	20	54	44	18

Positive control	AF-2	SA	AF-2	AF-2	9-AA	2-AA	2-AA	2-AA	2-AA	2-AA
S 9 mix	-					+				
Tester strain	TA100	TA1535	WP2uvrA	TA98	TA1537	TA100	TA1535	WP2uvrA	TA98	TA1537
N	259	231	217	231	259	253	231	213	227	249
Mean	510	527	220	529	603	986	221	935	562	216
S.D.	37	56	28	34	54	95	31	85	43	32
2S.D.	74	112	56	68	108	190	62	170	86	64
Mean-2S.D.	436	415	164	461	495	796	159	765	476	152
Mean+2S.D.	584	639	276	597	711	1176	283	1105	648	280

AF-2 : 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide, SA : Sodium azide, 9-AA : 9-Aminoacridine,

2-AA : 2-Aminoanthracene

Data collection period : October 1,1999~September 30,2000

PROJECT No. H-00266

QC STATEMENT OF COMPLIANCE

Title : Bacterial reverse mutation test of Agaro Oligosaccharide

PROJECT No. H-00266

Items of audit and inspection	Date of inspection	Date of reporting	
		Study director	Managing director
<b>Protocol</b>			
Draft	16. 10. 2000		
	17. 10. 2000	17. 10. 2000	17. 10. 2000
Final	19. 10. 2000	19. 10. 2000	19.10. 2000
<b>Dose selection test</b>			
Test substance preparation and administration	31. 10. 2000	01. 11. 2000	01. 11. 2000
Colony counting	02. 11. 2000	06. 11. 2000	06. 11. 2000
<b>Mutagenicity test</b>			
Test substance preparation and administration	10. 11. 2000	13. 11. 2000	13. 11. 2000
Colony counting	13. 11. 2000	17. 11. 2000	14. 11. 2000
<b>Raw data</b>			
	07. 12. 2000		
	11. 12. 2000	12. 12 .2000	11. 12 .2000
<b>Final report</b>			
Draft	07.12. 2000		
	11. 12. 2000	12. 12 .2000	11. 12 .2000
Final	15. 12. 2000	15. 12. 2000	15. 12. 2000

According to inspection, I, the undersigned, hereby confirm that this study has been conducted as per the protocol (GLP as reference) and the data are accurately reflected in this report.

Date: December 15, 2000.

In charge of QC

Satoru Sakamoto, D.V.M. (Impression of the seal)

Quality Assurance Unit

Nippon Experimental Medical Research Institute Co., Ltd.

Final Report

Title : Bacterial reverse mutation test of Agaro Oligosaccharide  
PROJECT No. H-00266

I the undersigned, hereby declare that the contents of this final report is confirmed by me.

Date: December 15, 2000.

Masaaki Kashima, D.V.M. (impression of the seal)

Managing director

Nippon Experimental Medical Research Institute Co., Ltd.

Contract Laboratory

## FINAL REPORT

Acute Oral Toxicity Study of Agaro Oligosaccharide in Rats

PROJECT No. H-00289

December 8, 2000

Nippon Experimental Medical Research Institute Co., Ltd.  
3303-58 Ohdo, Agatsuma-machi, Agatsuma-gun, Gunma-ken, Japan

## STATEMENT

Study Title: Acute Oral Toxicity Study of Agaro Oligosaccharide in Rats  
PROJECT No. H-00289

I, the undersigned, hereby declare that this report is the exact English version of the original report that written in Japanese language. Further, declare there is no difference in the contents of this report to that of the original (Japanese) report.

Study director: Iwao Kaneko.  
Translated by

Iwao Kaneko Date: 4/27/01

Iwao Kaneko  
Nippon Experimental Medical Research Institute Co., Ltd.

## APPROVAL OF FINAL REPORT

**Study Title: Acute Oral Toxicity Study of Agaro Oligosaccharide in Rats**  
**PROJECT No. H-00289**

I, the undersigned, hereby declare that this study was conducted in reference to the Japanese GLP Standards for Safety Studies on Drugs (Ministry of Health and Welfare of Japan, Ordinance No. 21: March 26, 1997).

Further this study has been conducted in accordance with the methods stated herein, and the data has been obtained from the study are accurately reflected in this final report.

Study Director: Iwao Kaneko                      <impression of seal>  
Date: December 8, 2000  
Nippon Experimental Medical Research Institute Co., Ltd.

## INTRODUCTION

**Study Title:** Acute Oral Toxicity Study of Agaro Oligosaccharide in Rats

**PROJECT No.** H-00289

### Objective

The study designed to assess the toxicity of Agaro Oligosaccharide following a single oral dose to rats.

### GLP standards

This study was conducted in reference to the Japanese GLP Standards for Safety Studies on Drugs (Ministry of Health and Welfare of Japan, Ordinance No. 21: March 26, 1997).

### Test guideline

This study was conducted in reference to the Amendments to the Single and Repeated Dose Toxicity Studies (YakuShinYaku No. 88: August 10, 1993)

### Sponsor

Takara Shuzo Co., Ltd.  
3-4-1 Seta, Otsu-shi, Siga-ken, Japan

### Contract Laboratory

Nippon Experimental Medical Research Institute Co., Ltd.  
3303-58 Ohdo, Agatsuma-machi, Agatsuma-gun, Gunma-ken, Japan

### Testing Facility

Haruna Laboratory  
Nippon Experimental Medical Research Institute Co., Ltd.  
3303-58 Ohdo, Agatsuma-machi, Agatsuma-gun, Gunma-ken, Japan  
Management: Masaaki Kashima

### Archives

#### Retention period:

A period specified in the provisions of the Article 26-2-3, Article 26-5(3),c, and Article 26-12, the Enforcement Regulations of the Pharmaceutical Affairs Law.

#### Items and location:

Protocol, raw data and final report (original) will be stored in raw data archive at Haruna Laboratory, Nippon Experimental Medical Research Institute Co., Ltd.  
Recods will be retained in record archive at Haruna Laboratory, Nippon Experi-

mental Medical Research Institute Co., Ltd.

### Study time schedule

Study initiation:	October 10, 2000
Animal receipt:	October 10, 2000
Grouping of animals:	October 11, 2000
Experiment initiation:	October 12, 2000
Administration:	October 12, 2000
Macroscopic examination:	October 26, 2000
Experiment termination:	October 26, 2000
Draft report:	November 17, 2000
Final report:	December 8, 2000
Study completion:	December 8, 2000

### Study personnel

Study direction, protocol preparation, work instructions and management, final report preparation :	Iwao Kaneko *
Animal health assessment :	Michiko Takahashi
Test substance management :	Akira Tomisawa
Test substance preparation :	Akira Tomisawa
Dosing, clinical observation and body weight measurement :	Akira Tomisawa, Kishio Hashizume, Tasaburo Hashizume, Yukihisa Karasawa
Macroscopic examination :	Akira Fukutome
Statistical analysis :	Yukio Tanaka, Masahiro Kasumi, Wataru Koike, Yukiko Takefuchi

\* : Safety Research Department, Nippon Experimental Medical Research Institute Co., Ltd.

**UNPREDICTED HAPPENINGS CONSIDERED TO HAVE  
AFFECTED THE RELIABILITY OF THE STUDY AND DE-  
VIATIONS FROM THE PROTOCOL**

**Study Title: Acute Oral Toxicity Study of Agaro Oligosaccharide in Rats**

**PROJECT No. H-00289**

There were no unpredicted happenings considered to have affected the reliability of the study and no deviations from the protocol.

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## 1. Summary

A group of ten SD rats [Crj:CD(SD)IGS] (five males and five females) received a single oral dose of Agaro Oligosaccharide at 0 (control) and 2000 mg/kg by gavage, in order to observe the signs of toxicity for 14 days and estimate LD50 value.

### 1.1 Clinical signs and mortality

There were no death and was no systemic response in any animal throughout the study.

### 1.2 Body weights

No differences in mean bodyweight on days 2, 3, 5, 8 and 15 after treatment were present between the control and treatment group.

### 1.3 Macroscopic findings

No treatment-related change was noted.

### 1.4 LD50 value

The LD50 value to either male or female rats of the test substance was demonstrated to be greater than 2000mg/kg bodyweight.

## 2. Materials and Methods

### 2.1 Test materials

#### 2.1.1 Test substance

Identity :	Agaro Oligosaccharide
Lot No. :	AG-0023-SD
Purity :	95%
Molecular weight :	Mean molecular weight of 1000
Physical State :	Off-white powder
Stability :	Stable under refrigeration
Date of manufacture :	May 25, 2000
Expiration date :	May 24, 2003
Storage conditions :	Under refrigeration, away from light, sealed
Sponsor :	Takara Shuzo Co., Ltd. 3-4-1 Seta, Otsu-shi, Siga-ken, Japan
Residual test substance :	All the remaining test substance was returned to the sponsor after completion of treatment.

### 2.1.2 Vehicle

Water for injection J.P. (Fuso Pharmaceutical Industries, Ltd., Lot No. 90901D; hereinafter referred to as water for injection)

## 2.2 Test substance preparation

### 2.2.1 Preparation method

A required amount of the test substance was weighed (adjusted for purity) and solubled in water for injection, at a concentration of 100mg/mL (w/v). The homogeneity of the test substance in the vehicle was visually checked and complete dissolution was confirmed. The stability in the vehicle was visually checked at preparation and just after administration, where no heat, coloration or foam was observed. Content analysis was not performed for the test substance formulation.

### 2.2.2 Time of preparation

The test substance was prepared on the day of dosing.

## 2.3 Animals and Environmental conditions

### 2.3.1 Animals

Pooled SD rats [Crj:CD(SD)IGS], 18 animals of each sex (body weights at delivery: 111-124g for males; 101-109g for females), were delivered at 5 weeks of age on September 29, 2000 from Atsugi Breeding Center, Charles River Japan Inc. (795 Shimofurusawa, Atsugi-shi, Kanagawa-ken). Then they were transferred on October 10, 2000 and acclimatized for 5 days prior to use. These rats were quarantined as pooled animals from September 29 to October 5, 2000. The animals were weighed during the quarantine period and assessed for health state on the last day of quarantine. They were acclimatized for 5 days, from the end of quarantine to the previous day of administration.

On the day of grouping, healthy 10 animals of each sex were selected and allocated to groups with computer system by stratified randomization based on the body weights taken on the day of grouping. The animals were 6 weeks old, and their body weight range was 188-206g for males and 138-157g for females on the day of administration.

### 2.3.2 Environmental conditions

Animals were individually housed in stainless bracket cages for rats (260W×380D×180Hmm) in an animal room (Room No.1 of Building E) where the environmental conditions were set as follows: temperature at  $22\pm 3^{\circ}\text{C}$ ; humidity at  $50\pm 20\%$  (actual values: temperature within the range of  $19\text{-}25^{\circ}\text{C}$ ; humidity within the range of 30-70%); ventilation frequency of at least 10 times per hour (all-fresh-air system); and lighting of 12 hours per day (from 6:00 a.m. to 6:00 p.m., 150-300 lux). Animals were allowed free access to pellet feed for experimental animals (CE-2, Lot No. E2080,

Clea Japan Inc.) and drinking water (household tap water). Cages, feeders, trays and watering bottles were autoclaved (121°C for 30 min) prior to use. Watering bottles and trays were changed at least twice weekly. The animal room was cleaned after work every day, and the floor was sterilized by wiping with 400-fold dilution of benzethonium chloride (Hyamine, Sankyo Co., Ltd.).

Identification was made for animals by writing abbreviated animal numbers on the root of the tail with an oil marker, and for cages by attaching colored labels showing study No., administration route, dose levels, etc.

### 2.3.3 Analysis of impurities and contaminants in diet and water

For impurities and contaminants in the diet, we obtained a copy of the results of the analysis that was conducted by Tokyo Kenbikyo-in (44-1, Hakozaki-cho, Nihonbashi, Chuo-ku, Tokyo) at the request of the manufacturer. Checked before the diet was served, the analytical results were confirmed to be within the range of acceptable limits specified by our facility. For impurities and contaminants in the drinking water, the analysis was conducted by the Environmental Hygiene Laboratory Center of Gunma Pharmaceutical Association (5-18-36 Nishikatagai-cho, Maebashi-shi, Gunma-ken) based on the Ministerial Ordinance Concerning Water Quality Standards (Ministry of Health and Welfare of Japan, Ordinance No. 69, 1992) with the water samples periodically collected by our facility. The types of analyses included clean water testing on standard parameters (August 3, 1999) trihalomethan test (August 8, 2000), and the tests pursuant to the building management law on all parameters (February 15, 2000) and with omitted parameters (September 6 and October 3, 2000). All the analytical results were within the above water quality standards, there being no abnormal values considered to have affected the study.

## 2.4 Constitution of the groups, dose levels and rationale for dose selection

### 2.4.1 Constitution of the groups and dose Levels

The constitution of the groups and the treatment regime are indicated in the following table.

Group No.	Test materials	Dose level (mg/kg)	Dose volume (mL/kg)	Concentration (mg/mL)	Sex	No. of animals	Animal No.
00	Vehicle (Control <sup>a</sup> )	0	20	0	male	5	00M01~00M05
					female	5	00F01~00F05
01	Agaro Oligo-saccharide	2000	20	100	male	5	01M01~01M05
					female	5	01F01~01F05

a : The controls received water for injection.

#### 2.4.2 Rationale for dose selection

The dose level for the main study was chosen on the basis of a preliminary study (PROJECT No.H-00377). In the preliminary study, a group of four SD rats [Crj:CD(SD)IGS] (two males and two females) received a single oral dose of Agaro Oligosaccharide at 500, 1000 and 2000 mg/kg by gavage. As there were no deaths even at the highest dose of 2000 mg/kg, in compliance with the manual of the Guideline for Medical Devices (1997), the limit dose of 2000 mg/kg was selected for the main study

#### 2.5 Administration

##### 2.5.1 Route of administration and rationale for the selection

Oral administration was selected because oral intake is expected as a route of exposure in humans.

##### 2.5.2 Method of administration

Dose volume was adjusted to the body weights taken just before administration, and administered with a 5mL disposable syringe connected to a gavage.

##### 2.5.3 Administration frequency and rationale for the selection

A single administration was selected in accordance with the Guideline for Medical Devices (Yaku ShinYaku No.88). Animals were fasted from about 18 hours before to about 3 hours after treatment.

#### 2.6 Observation, measurement and examination

The day of treatment was designated as day 0, and the following observation, measurement and examination were performed on all animals up to day 15 after treatment.

##### 2.6.1 Clinical signs

Animals were observed for clinical signs and mortality at 15, 30 minutes, 1, 3, and 6 hours after treatment on day 0 and thereafter once daily up to day 15.

##### 2.6.2 Body weights

Animals were weighed with an electronic balance (Sartrius Co., Ltd.) on day 0 (just before treatment), and on days 2, 3, 5, 8 and 15 after treatment.

##### 2.6.3 Macroscopic examination

All animals were killed by transecting the abdominal aorta under ether anesthesia at the end of the observation period (day 15 after treatment) and subjected to a macroscopic examination that consisted of opening the cranial, thoracic and abdominal cavities. The macroscopic appearance of all examined organs was recorded.

#### 2.6.4 Microscopic examination

Microscopic examination was not performed because no abnormal organs were found in macroscopic examination.

#### 2.7 Statistical analysis

Means and standard deviations of the body weights were calculated for the test substance group and the vehicle control. The F-test for homogeneity of variances was performed on the data. If the F-test indicated homogeneous variances, the group means were compared to the vehicle control means using Student's t-test. If the F-test indicated heterohomogeneous variances, Aspin-Welch's t-test was used to compare group to the vehicle control.

### 3. Results

#### 3.1 Mortality and clinical signs (Table 1~4, Appendices 1 and 2)

There were no deaths and was no systemic response in any animal throughout the study.

#### 3.2 Body weights (Fig. 1 and 2, Tables 5 and 6, Appendices 3 and 4)

A decrease in body weights was recorded for one female of the test substance group on day 3 after treatment, but the other animals demonstrated satisfactory body weight gains. No differences in mean bodyweight on days 2, 3, 5, 8 and 15 after treatment were present between the control and treatment group.

#### 3.3 Macroscopic findings (Tables 7 and 8, Appendices 5 and 6)

No treatment-related change was noted

### 4. Discussion and Conclusion

There were no deaths and was no systemic response in any animal throughout the 14-day observation period. No differences in mean bodyweight on days 2, 3, 5, 8 and 15 after treatment were present between the control and treatment group. No abnormalities were recorded at the macroscopic examination. Therefore, Agarose Oligosaccharide at a dose of 2000mg/kg did not produce toxic effect in SD rats.

The LD50 value to either male or female SD rats of Agarose Oligosaccharide was demonstrated to be greater than 2000mg/kg bodyweight.

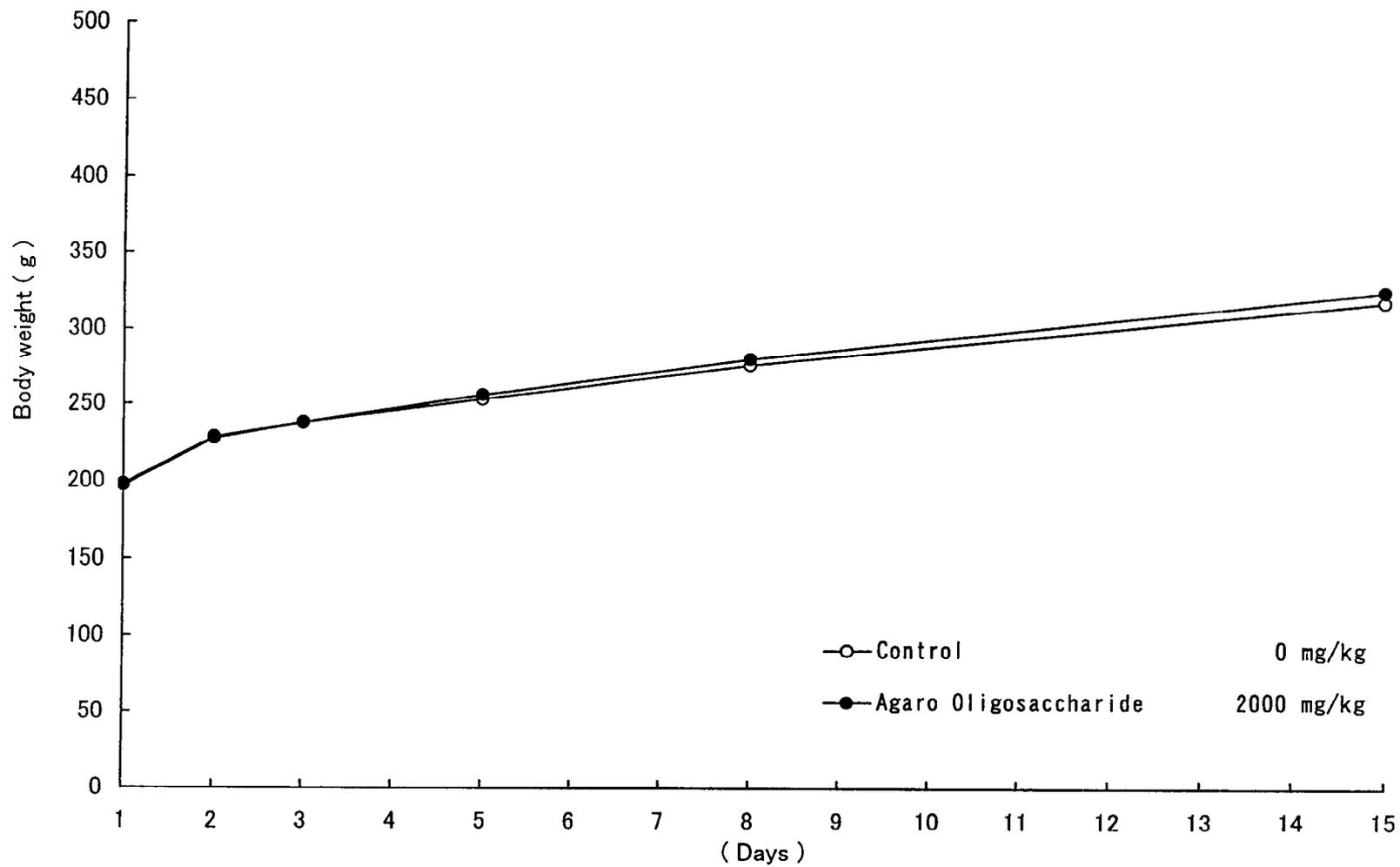


Fig.1 Body weight changes of male rats treated orally with Agaro Oligosaccharide

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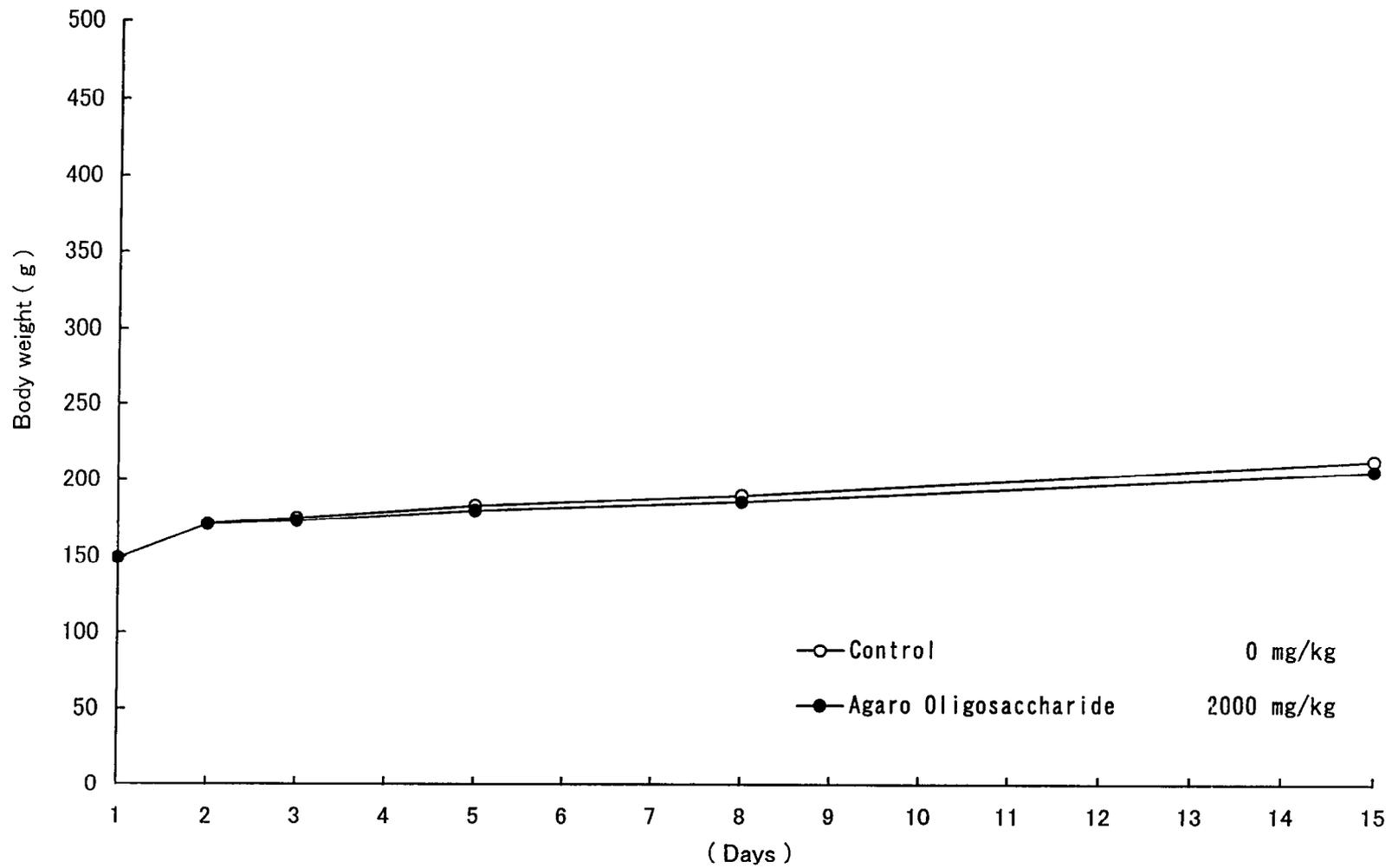


Fig.2 Body weight changes of female rats treated orally with Agaro Oligosaccharide

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Table 1 Mortality of male rats treated orally with Agaro Oligosaccharide

Group No.	Dose (mg/kg)	No. of death	Days																	Mortality		
			1		2			3	4	5	6	7	8	9	10	11	12	13	14		15	
			min		h																	
			15	30	1	3	6															
00	Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
01	Agaro Oligosaccharide	2000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5

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Table 2 Mortality of female rats treated orally with Agaro Oligosaccharide

Group No.	Dose (mg/kg)	No. of death	Days																	Mortality	
			1		2		3	4	5	6	7	8	9	10	11	12	13	14	15		
			min		h																
			15	30	1	3	6														
00	Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5
01	Agaro Oligosaccharide	2000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/5

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Table 3

Clinical signs of male rats treated orally with Agaro Oligosaccharide

Group-No. Dose (mg/kg)	Findings	< Days >																	
		1		2			3	4	5	6	7	8	9	10	11	12	13	14	15
		min		h															
		15	30	1	3	6													
00 Control 0	No. of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	
	no abnormality	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	
01 Agaro Oligosaccharide 2000	No. of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	
	no abnormality	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	

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Table 4

Clinical signs of female rats treated orally with Agaro Oligosaccharide

Group-No. Dose (mg/kg)	Findings	< Days >																	
		1		2			3	4	5	6	7	8	9	10	11	12	13	14	15
		min		h															
		15	30	1	3	6													
00 Control 0	No. of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	no abnormality	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
01 Agaro Oligosaccharide 2000	No. of animals	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	no abnormality	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5

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Table 5

## Body weights of male rats treated orally with Agaro Oligosaccharide

Group-No.		.....< D a y s >.....					
Dose (mg/kg)		1	2	3	5	8	15
00	N	5	5	5	5	5	5
Control	Mean	197	228	238	253	276	318
0	S.D.	7	9	10	13	13	17
01	N	5	5	5	5	5	5
Agaro Oligosaccharide	Mean	198	229	238	256	280	325
2000	S.D.	6	6	7	7	8	19

Unit : g      N : No. of animals

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Table 6

Body weights of female rats treated orally with Agaro Oligosaccharide

Group-No. Dose (mg/kg)		.....< D a y s >.....						
		1	2	3	5	8	15	
00 Control 0	N	5	5	5	5	5	5	
	Mean	149	171	175	183	190	213	
	S.D.	3	6	9	6	8	8	
01 Agaro Oligosaccharide 2000	N	5	5	5	5	5	5	
	Mean	149	171	173	180	186	206	
	S.D.	7	8	11	7	10	11	

Unit : g      N : No. of animals

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Table 7 Necropsy of male rats treated orally with Agaro Oligosaccharide

Organs / Findings	Group No.	00	01
	Dose	Control	Agaro Oligosaccharide
	(mg/kg)	0	2000
	No. of animals	5	5
abnormality		0	0

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Table 8 Necropsy of female rats treated orally with Agaro Oligosaccharide

Organs / Findings	Group No.	00	01
	Dose (mg/kg)	Control	Agaro Oligosaccharide
		0	2000
	No. of animals	5	5
abnormality		0	0

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## Appendix 1

## Clinical signs of male rats treated orally with Agaro Oligosaccharide

Group-No. Dose (mg/kg)	Animal No.	< Days >																	
		1		2			3	4	5	6	7	8	9	10	11	12	13	14	15
		min		h															
		15	30	1	3	6													
00	00M01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Control 0	00M02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	00M03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	00M04	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	00M05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	01	01M01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Agaro Oligosaccharide 2000	01M02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	01M03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	01M04	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	01M05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- : no abnormality

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Appendix 2

Clinical signs of female rats treated orally with Agaro Oligosaccharide

Group-No. Dose (mg/kg)	Animal No.	< Days >																			
		1		2	3	4	5	6	7	8	9	10	11	12	13	14	15				
		min		h																	
		15	30	1	3	6															
00 Control 0	00F01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	00F02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	00F03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	00F04	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	00F05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
01 Agaro Oligosaccharide 2000	01F01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	01F02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	01F03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	01F04	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
	01F05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			

- : no abnormality

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## Appendix 3

## Body weights of male rats treated orally with Agaro Oligosaccharide

Group-No.	.....< D a y s >.....						
Dose (mg/kg)	Animal No.	1	2	3	5	8	15
00	00M01	196	226	235	252	276	322
Control 0	00M02	206	240	253	270	294	334
	00M03	203	234	242	263	281	323
	00M04	191	217	226	236	257	289
	00M05	190	221	233	246	272	322
	N	5	5	5	5	5	5
	Mean	197	228	238	253	276	318
S. D.	7	9	10	13	13	17	
01	01M01	203	234	244	260	284	329
Agaro Oligosaccharide 2000	01M02	200	229	241	258	279	325
	01M03	188	218	226	244	269	302
	01M04	202	232	235	257	275	318
	01M05	199	232	242	262	291	353
	N	5	5	5	5	5	5
	Mean	198	229	238	256	280	325
S. D.	6	6	7	7	8	19	

Unit : g      N : No. of animals

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## Appendix 4

## Body weights of female rats treated orally with Agaro Oligosaccharide

Group-No. Dose (mg/kg)	Animal No.	D a y s					
		1	2	3	5	8	15
00 Control 0	00F01	149	172	175	186	192	212
	00F02	146	165	165	178	177	204
	00F03	152	176	182	187	192	214
	00F04	146	166	168	176	189	208
	00F05	152	178	186	188	198	225
	N	5	5	5	5	5	5
	Mean	149	171	175	183	190	213
S.D.	3	6	9	6	8	8	
01 Agaro Oligosaccharide 2000	01F01	157	182	187	190	198	222
	01F02	138	161	157	171	172	191
	01F03	149	175	175	179	182	206
	01F04	148	168	169	180	184	200
	01F05	155	168	177	182	192	209
	N	5	5	5	5	5	5
	Mean	149	171	173	180	186	206
S.D.	7	8	11	7	10	11	

Unit : g      N : No. of animals

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Appendix 5 Necropsy of male rats treated orally with Agaro Oligosaccharide

Group No.	00					01				
Dose (mg/kg)	Control					Agaro Oligosaccharide				
Animal No.	0					2000				
	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	1	1	1	1	1
	M	M	M	M	M	M	M	M	M	M
	0	0	0	0	0	0	0	0	0	0
Organs / Findings	1	2	3	4	5	1	2	3	4	5
abnormality	-	-	-	-	-	-	-	-	-	-

- : Negative

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Appendix 6 Necropsy of female rats treated orally with Agaro Oligosaccharide

Group No.	00					01				
Dose (mg/kg)	Control					Agaro Oligosaccharide				
	0					2000				
Animal No.	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	1	1	1	1	1
	F	F	F	F	F	F	F	F	F	F
Organs / Findings	0	0	0	0	0	0	0	0	0	0
abnormality	1	2	3	4	5	1	2	3	4	5
	-	-	-	-	-	-	-	-	-	-

- : Negative

PROJECT No. H-00289

## CONFIRMATION

**Study Title: Acute Oral Toxicity Study of Agaro Oligosaccharide in Rats**  
**PROJECT No. H-00289**

I, the undersigned, have confirmed the contents of the final report of the above study.

**Management: Masaaki Kashima**      <impression of seal>

**Date: December 8, 2000**

**Nippon Experimental Medical Research Institute Co., Ltd.**

## QC CONFIRMATION

Study Title: Acute Oral Toxicity Study of Agaro Oligosaccharide in Rats  
PROJECT No. H-00289

Study Phase	Date of inspection	Date of Reporting	
		Study Director	Management
Protocol (draft)	September 22, 25,2000	September 26, 2000	Septembet 25, 2000
Protocol (final)	October 11, 2000	October 11, 2000	October 11, 2000
Process Base Inspections			
Weights of animals, test substance preparation, administration, and clinical observation			
	October 12, 2000	October 16, 2000	October 13, 2000
Macroscopic finding	October 26, 2000	October 31, 2000	October 30, 2000
Raw data	November 27, 28, 2000	November 28, 2000	November 28, 2000
Report (draft)	November 27, 28, 2000	November 28, 2000	November 27, 2000
Report (final)	December 8, 2000	December 8, 2000	December 8, 2000

I, the undersigned, have confirmed the concordance of study operations with the protocol, and the consistency of the final report with the raw data.

Satoru Sakamoto <impression of seal>

QC inspector (Quality Assurance Unit Manager)

Date: December 8, 2000

Nippon Experimental Medical Research Institute Co., Ltd.

*Japanese Journal of Cancer Research Vol. 90, P151 (1999)*

**Anti-inflammatory action of Agaro-oligosaccharides on TPA-induced inflammation in mice**

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**Introduction**

Metabolites of arachidonic acid play a major role in the occurrence of inflammation and pain in tissue. Arachidonic acid derived from phospholipids in the cell membrane is metabolized to form prostaglandins, prostacyclins and thromboxane by the action of cyclooxygenase. Prostaglandins have vasodilator and associated blood flow increasing action, but the action of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is closely connected with increases in edema and leukocyte infiltration.

By topically application to the skin with 12-o-tetradecanoylphorbol-13-acetate (TPA), a potent promoter of carcinogenesis, metabolism of arachidonic acid in the cells at the application site is promoted and chemicals such as PGE<sub>2</sub> are produced<sup>1)</sup>. These inflammatory substances promote vascular permeability and skin edema occurs. It is known that inflammation caused by continuous stimulation with TPA is involved in carcinogenesis<sup>2)</sup>. In the course of carcinogenesis, there is an initiation stage of mutagenicity that damages the cellular DNA and a promotion stage with loss of control of cell proliferation, and these stages are considered necessary of carcinogenesis. 7,12-Dimethylbenz[a]anthracene (DMBA) acts as an initiator and TPA acts as a promoter for carcinogenesis. The promoter action of TPA is considered to be due to the induction inflammation in the body as follows. TPA causes release of inflammatory mediators such as PGE<sub>2</sub><sup>3)</sup> and nitric oxide (NO)<sup>4)</sup> from inflammatory cells such as macrophages (Mφ). PGE<sub>2</sub> selectively activates type 2 helper T-cells (Th2 cells) and the activated Th2 cells inhibit the type 1 helper T-cells (Th1 cells) which play an important role in tumor rejection<sup>5)</sup>. However, NO also directly inhibits Th1 cells<sup>6)</sup>. The body's defense mechanism against cancer is suppressed by the inhibition of Th1 cells by PGE<sub>2</sub> and NO, and carcinogenesis is induced. To date, it has been reported

that carcinogenesis is inhibited by non-steroidal anti-inflammatory drugs (NSAIDs)<sup>7)</sup> or steroids<sup>8)</sup> that inhibit PGE<sub>2</sub> production. In the present study, we investigated the effects of agar-derived oligosaccharides on inflammation and carcinogenesis induced by TPA.

## Materials and Methods

### Effects on NO and PGE<sub>2</sub> production by mouse peritoneal macrophages

ddY Mice (females, 7 weeks of age, Japan SLC) were administered 10% agaro-oligosaccharides *ad libitum* in drinking water for 21 days. Then 4 mL of RPMI-1640 medium (BioWhittaker) was injected into the peritoneal cavity of mice sacrificed by exsanguination, the peritoneal cavity was washed and peritoneal cells were recovered. The cells were suspended in RPMI-1640 medium containing 10% fetal bovine serum (Hyclone), the cell concentration was adjusted to 10<sup>6</sup> cells/mL and the suspension was inoculated into a 48-well microtiter plate at 500  $\mu$ l/well. After incubation for 2 hours in a CO<sub>2</sub> incubator at 37°C, the adherent cells were used as peritoneal macrophages. Lipopolysaccharide (LPS, Sigma) at 5  $\mu$ g/mL and interferon- $\gamma$  (IFN- $\gamma$ , CosmoBio) at 2,000 U/mL were added and the plates were incubated for 12 hours in a CO<sub>2</sub> incubator at 37°C. After completion of incubation, the concentration of NO in the supernatant was obtained by measuring the amount of NO<sub>2</sub><sup>-</sup>, an oxidation product of NO. This measurement was made by adding 100  $\mu$ L of 4% Griess reagent (Sigma) to 100  $\mu$ L of supernatant and measuring the absorbance at 490 nm after 15 min. The amount of PGE<sub>2</sub> in the medium supernatant at 12 hours after the addition of 50  $\mu$ g/mL of LPS to the peritoneal macrophages was measured using an ELISA kit (Neogen).

### Effects on TPA-induced edema and PGE<sub>2</sub> production in mouse ear

The entire external surface of the right ear of ICR mice (females, 7 weeks of age, weighing about 25 g; Japan SLC) was topically treated with a 5 nmol/20  $\mu$ L TPA acetone solution. The mice were divided into groups of three animals each and the amount of PGE<sub>2</sub> in the ear at 2 hours after TPA application and the ear edema at 6 hours after application were measured. PGE<sub>2</sub> measurement was performed by excising the entire auricle from mice sacrificed by exsanguination. The skin sheets were homogenized in 500  $\mu$ L of extract (100 nmol/L Tris HCl buffer solution, 1 mmol/L

EDTA, 2 nmol/L reduced glutathione and 2  $\mu$ mol/L hemoglobin). The supernatant was obtained by centrifugation at 10,000 G for 10 min. Then 500  $\mu$ L of 80% ethanol and 10  $\mu$ L of glacial acetic acid were added to the supernatant, and the supernatant was recovered by centrifugation at 2,500 G for 5 min after letting stand for 5 min. The recovered supernatant was applied to a C18 column (Waters Co.) and eluted with 2 mL of water and 2 mL of hexane. After adding 4 mL of ethyl acetone containing 1% methanol, the ethyl acetone layer was recovered and evaporated to dryness in nitrogen gas. The amount of PGE<sub>2</sub> in the extract was measured using an ELISA kit. Ear edema was measured by excising the entire ear on the TPA application side from mice sacrificed by exsanguination and weighing it. Administration of agaro-oligosaccharides was performed by placing 10% agaro-oligosaccharides in the drinking water bottle and supplying water *ad libitum* from 14 days before TPA application until sacrifice.

#### Carcinogenesis inhibitory action in a mouse skin 2-stage carcinogenesis model

ICR mice (females, 9 weeks of age, weighing about 30 g; Japan SLC) were divided into groups of 10 animals each for each experiment. The dorsal hair of each mouse was shaved and initiation was performed by applying 100  $\mu$ g of DMBA (Sigma) dissolved in 100  $\mu$ L of acetone. From after 1 week, promotion was performed by continuing to apply 1  $\mu$ g of TPA (Nakarai Tesque) dissolved in 100  $\mu$ L of acetone to the same site twice a week for 20 weeks. Administration of agaro-oligosaccharides was performed by placing 1% or 3% agaro-oligosaccharides in the drinking water bottle and supplying water *ad libitum* from 7 days before DMBA application. For 20 weeks after the start of TPA application, the number of papillomas formed on the back of each mouse and the number of mice with papillomas were determined once a week.

#### Statistical analysis

Significant differences from the controls were tested by the Mann Whitney and Student's t-test, and  $p < 0.05$  was taken as statistically significant.

## Results

#### Effects on NO and PGE<sub>2</sub> production by mouse peritoneal macrophages

Stimulation of mouse peritoneal macrophages with LPS and IFN-gamma resulted

in NO production about four times higher than that in non-stimulated controls. Stimulation with LPS also caused PGE<sub>2</sub> production to increase by about 50 fold. However, in mice given drinking water containing agaro-oligosaccharides, the NO production was reduced to normal levels and PGE<sub>2</sub> production was also markedly inhibited (Fig. 1). Administration of agaro-oligosaccharides inhibited activation of macrophages.

#### Effects on TPA-induced edema and PGE<sub>2</sub> production in mouse ear

In the control group with TPA application, PGE<sub>2</sub> production was increased by about three fold when compared with normal mice. In the group administered agaro-oligosaccharides, PGE<sub>2</sub> was significantly reduced compared with the controls and it was evident that PGE<sub>2</sub> production was inhibited by localized inflammation caused by agaro-oligosaccharides (Fig. 2a).

In the control group with TPA applied to the ears, the ear weight increased by at least double, but in the agaro-oligosaccharide group, the ear weight was significantly decreased when compared with the controls, showing that agaro-oligosaccharides have inhibitory effects on edema (Fig. 2b).

#### Carcinogenesis inhibitory action in a mouse skin 2-stage carcinogenesis model

In the control group, partial tumorigenesis was observed at Week 7 after the start of TPA application. At Week 11, all mice showed tumorigenesis and the average number of papillomas was 18.3 at Week 20. However, in the group administered agaro-oligosaccharides in the drinking water, carcinogenesis was inhibited dose-dependently and the average number of papillomas at Week 20 was 7.4 with 1% and 4.4 with 3% agaro-oligosaccharide administration. In the controls, all mice had tumors at Week 11, but no tumors were found after 20 weeks in two mice given 1% and three given 3% agaro-oligosaccharides (Fig. 3).

### Discussion

In mice given drinking water containing agaro-oligosaccharides, the PGE<sub>2</sub> and NO production in LPS or IFN- $\gamma$ -stimulated cultures of peritoneal macrophages were inhibited. Furthermore, agaro-oligosaccharides markedly inhibited TPA-induced PGE<sub>2</sub>

production and consequently inhibited ear edema. It was suggested that agaro-oligosaccharides manifest anti-inflammatory action by inhibiting production of PGE<sub>2</sub> and NO, important mediators in the induction of inflammation. In a two-stage skin carcinogenesis model using DMBA and TPA, tumorigenesis was markedly inhibited in mice given agaro-oligosaccharides in their drinking water.

Activation of Th2 cells by PGE<sub>2</sub> is inhibited by the inhibitory action of agaro-oligosaccharides on PGE<sub>2</sub> production by inflammatory cells and this in turn suppresses inhibition of Th1 cells by Th2 cells. Inhibition of NO production also appears to suppress inhibition of Th1 cells directly by NO. Therefore, it is assumed possible that inhibition of PGE<sub>2</sub> and NO production by agaro-oligosaccharides prevents reduction of the cellular immune response for antitumor immune responses and suppresses tumorigenesis.

In addition to inhibition of Th1 cells, PGE<sub>2</sub> and NO have other actions including carcinogenesis in normal cells and promotion of growth of cancer cells<sup>9,10</sup>). Agaro-oligosaccharides also appear to inhibit direct carcinogenic action of PGE<sub>2</sub> and NO.

In inflammatory diseases, arachidonic acid metabolites are involved in skin diseases classified as acute urticaria including erythema and flare response. TPA skin disease models are also used as psoriasis models because of the histological similarities<sup>11</sup>). However, it is known that NO is important in the onset of autoimmune diseases such as rheumatism<sup>12</sup>) and agaro-oligosaccharides that inhibit production of PGE<sub>2</sub> and NO are considered effective against such inflammatory diseases.

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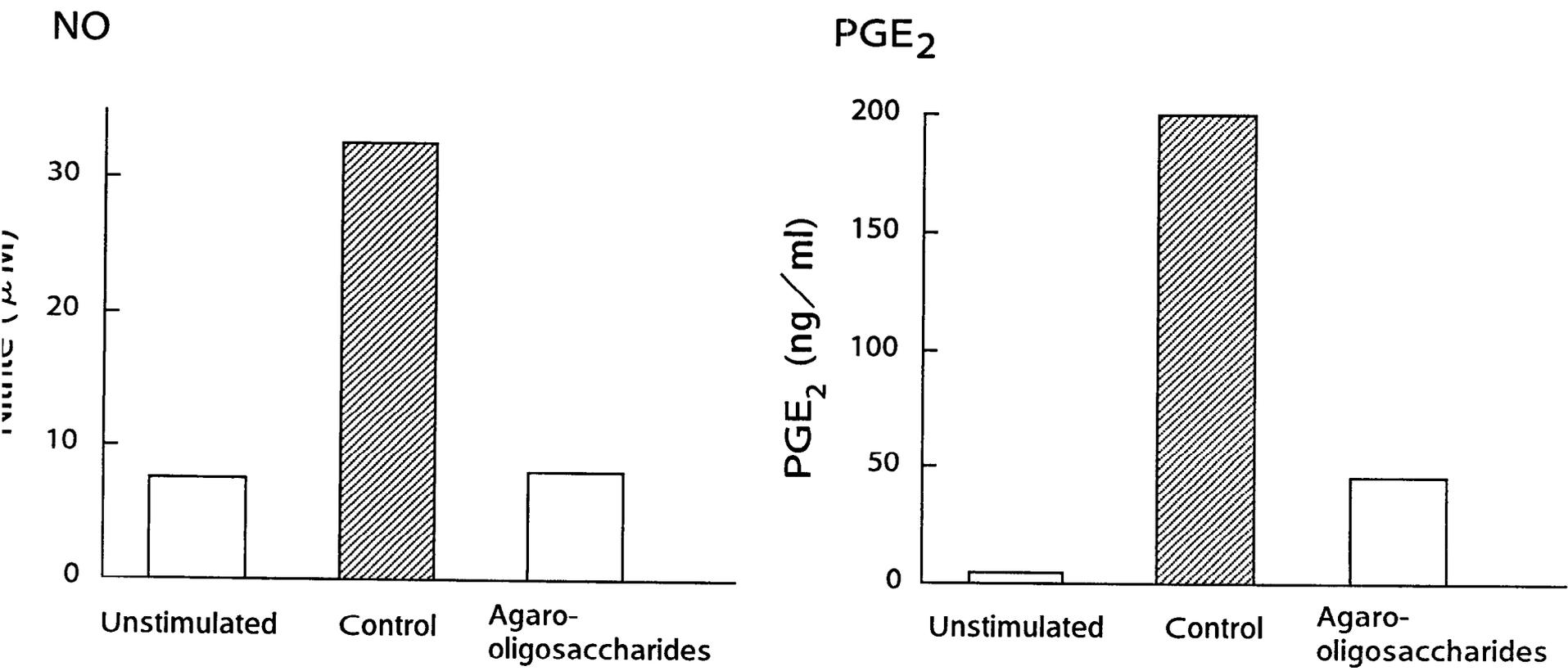
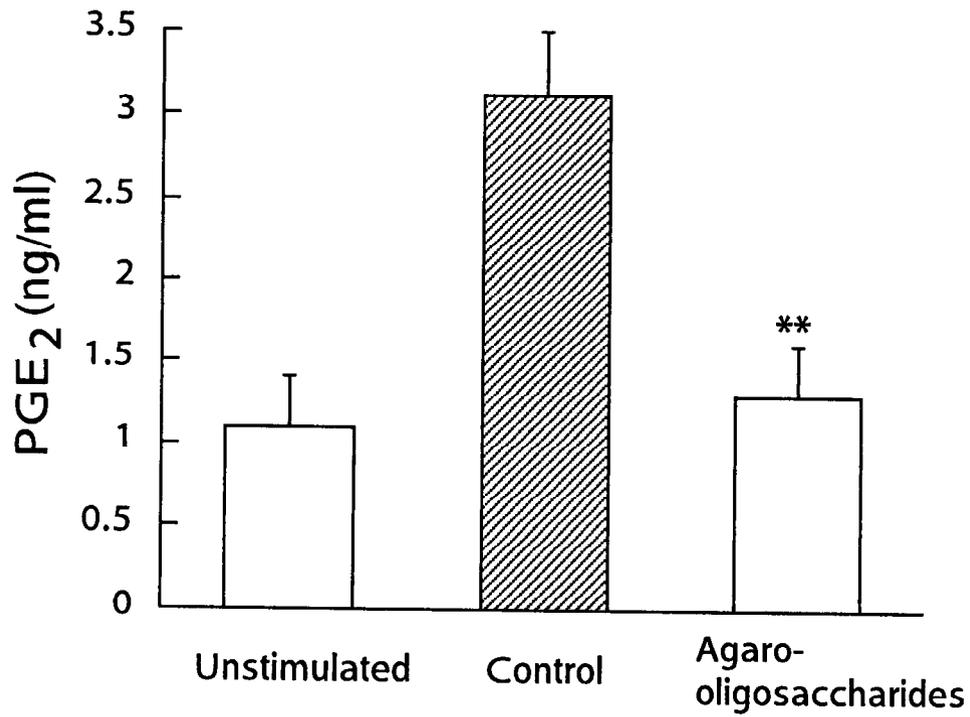
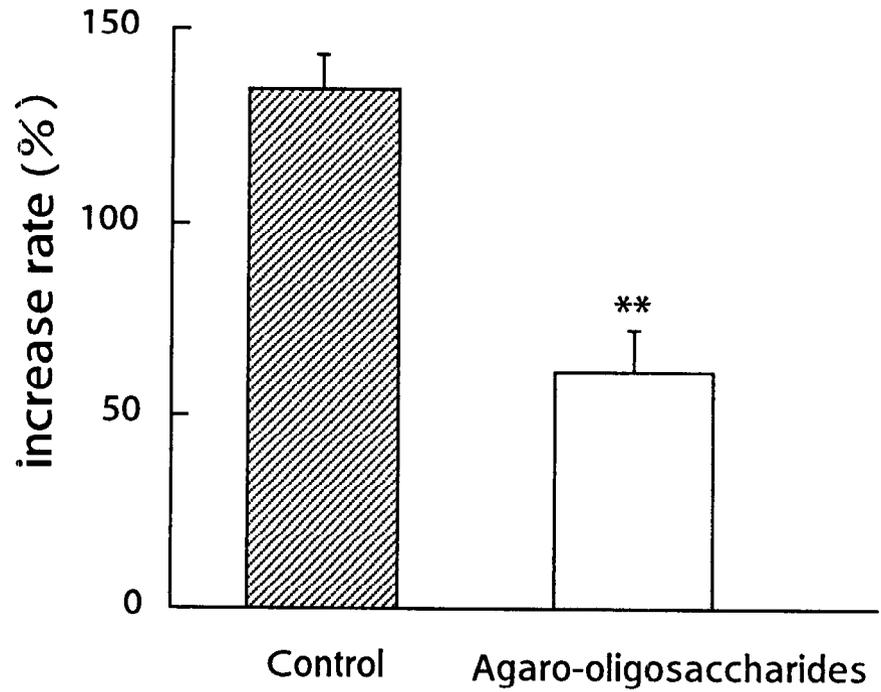


Fig. 1 Effect of NO and PGE<sub>2</sub> production by mouse peritoneal Mφ

a. PGE<sub>2</sub>

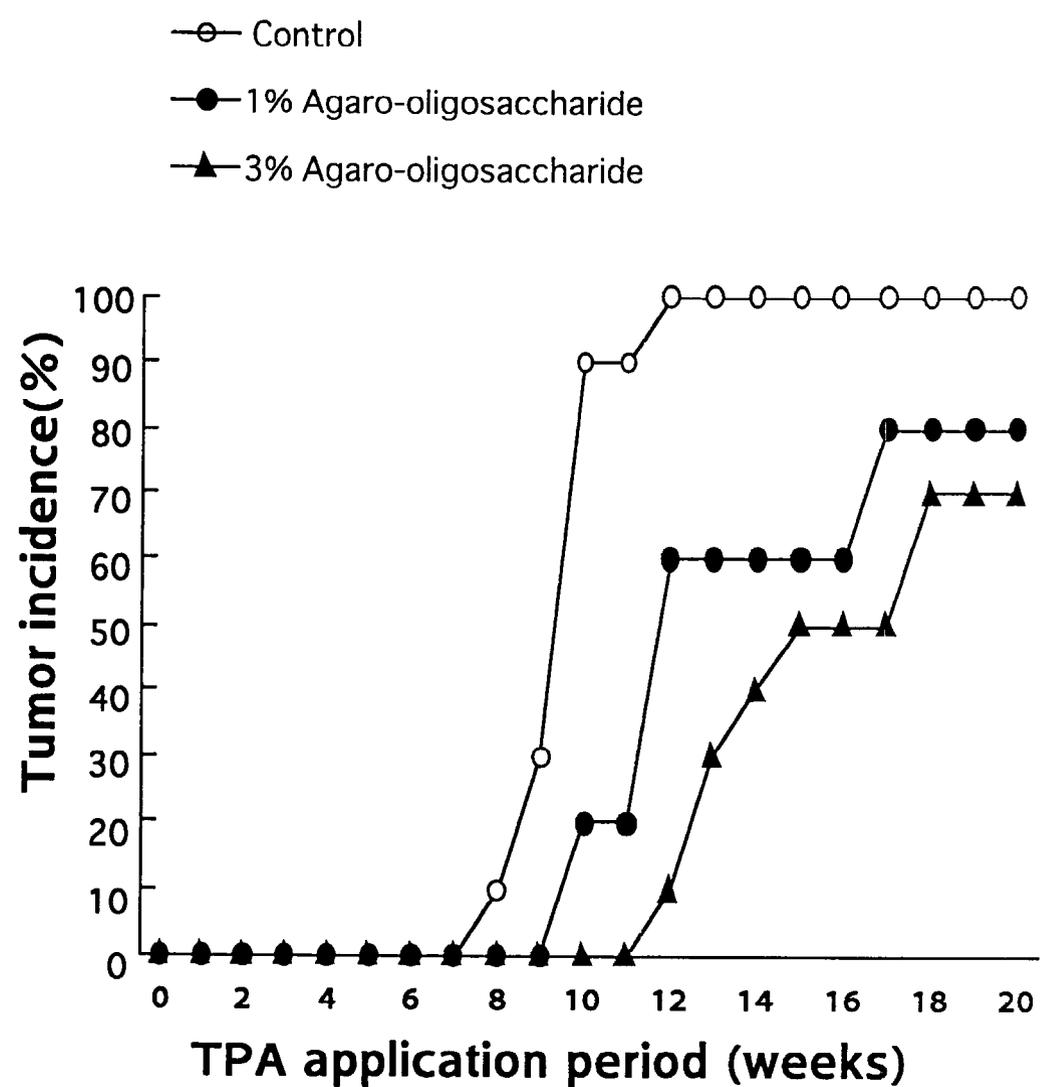
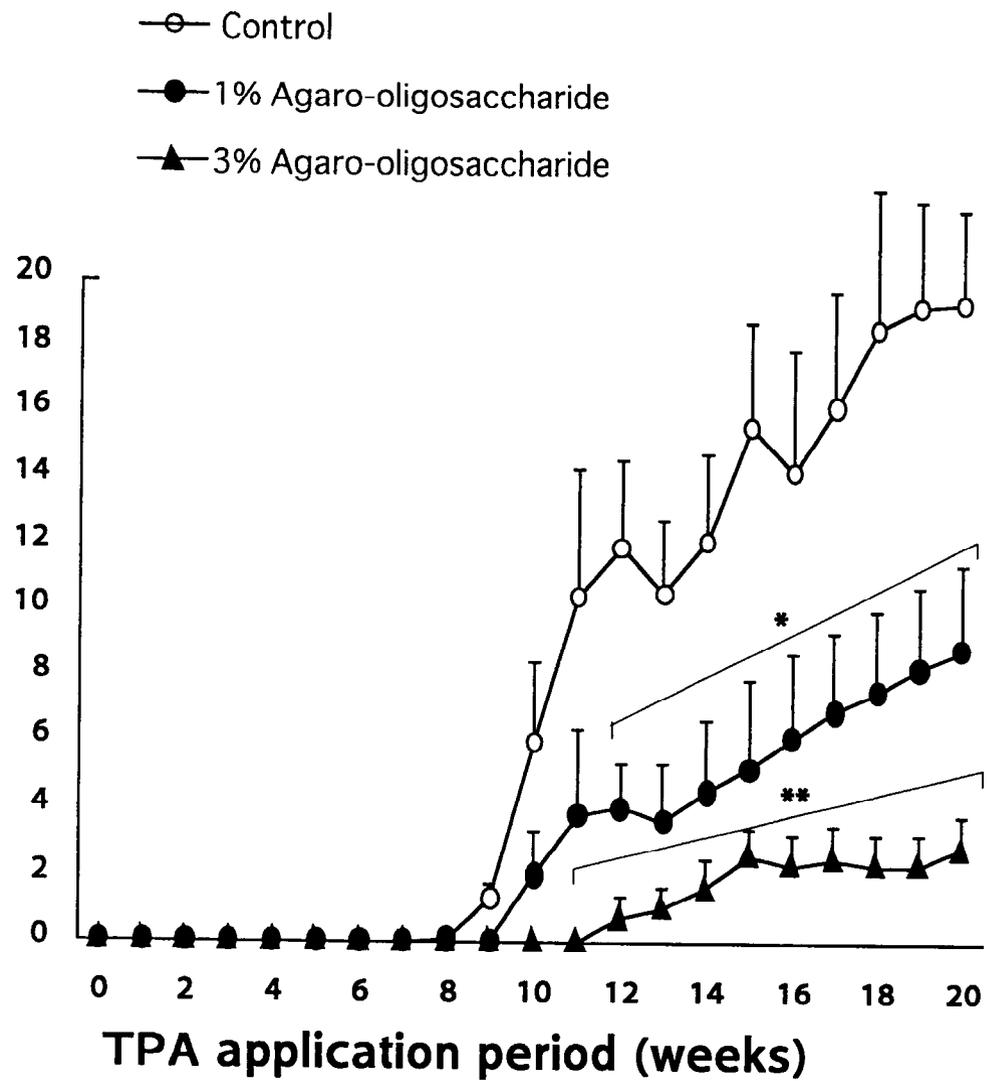


b. Edema



Data show mean  $\pm$  SEM of 3 animals.  
\*\*:  $p < 0.01$  vs control

Fig. 2 Effects of TPA-induced edema and PGE<sub>2</sub> production in mouse ear



Data show mean  $\pm$  SEM of 10 animals.

\*:  $p < 0.05$ , \*\*:  $p < 0.01$  vs control

**Fig.3 Carcinogenesis inhibitory action in a mouse skin 2-stage carcinogenesis model**

*The Japanese Biochemical Society, vol 171, 703 (1999)*

## **Therapeutic and preventive effects of Agaro-oligosaccharides on acute and chronic inflammation in mice**

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### **<Introduction>**

We have previously reported that Agaro-oligosaccharides inhibits the proliferation of tumor cells *in vitro* and also inhibits the production of NO from macrophages induced by LPS stimulation in mice.

In this report, we studied the anti-inflammatory effects of the Agaro-oligosaccharides *in vivo* using mouse experimental models. We studied its effect to [I] improve the mortality and inhibit TNF- $\alpha$  production in the endotoxin-induced shock and fatal fulminant hepatitis models and [II] inhibit the induction of the chronic rheumatoid arthritis disease model of type II collagen-induced arthritis in mice.

### **<Materials and methods>**

#### **[I] Endotoxin shock and fatal fulminant hepatitis model**

CDF1 mice (female, 6 weeks of age, Japan SLC., Inc.) were provided freely with drinking water containing Agaro-oligosaccharides at the agar concentration of 10%, 5%, or 1%. Tap water was used to make the dilutions. The control group was provided with tap water.

(1) At Day 19 after the start of the consumption of the drinking water, a high dose of LPS (Sigma) was administered intraperitoneally (300  $\mu\text{g}/\text{mouse}$ ) to prepare the fatal endotoxin shock model, and the inhibition of the mortality to 72 hr was determined.

(2) To the mice similarly provided with the drinking water, the mice were treated intraperitoneally on Day 19 with a low dose of LPS (20  $\mu\text{g}/\text{mouse}$ ), and 1 hr later the serum TNF- $\alpha$  concentration measured using a commercial ELISA kit (R&D Co.).

(3) To prepare the endotoxin-induced liver injury model, the mice were administered intraperitoneally with galactosamine (Sigma) (20 mg/mouse) and LPS (0.01  $\mu\text{g}/\text{mouse}$ ) simultaneously to prepare a model of fatal fulminant hepatitis, and the effect on survival

was measured<sup>1)</sup>.

## [II] Mouse type II collagen induced arthritis model

Bovine cartilage-derived type II collagen (K41/Collagen Gijyutsu-kenshubai) at 3 mg/mL and equal amount of Freund's complete adjuvant (FCA, Difco Co.) were mixed to obtain an emulsion, which was injected subcutaneously into DBA/1 J mice (males, 6 weeks of age, Seac Yoshitomi, Ltd.) at the base of the tail (150  $\mu$ g/0.1 mL/mouse). 3 weeks later, a booster injection was given to the mice to induce the type II collagen-induced arthritis<sup>2)</sup>. The Agaro-oligosaccharides were dissolved in tap water to give concentrations of 3% and 0.3% and was provided *ad libitum* from the time of initial collagen sensitization [Assessment of Preventive Effect] or from the time of booster immunization [Assessment of Therapeutic Effect]. The control group was provided *ad libitum* with tap water.

There were 10 animals per experimental group. The degree of joint inflammation was evaluated by a scoring system for all limbs, in which no change was scored as 0 points, edema in 1 or more digits was scored as 1 point, erythema and diffuse edema were scored as 2 points, severe diffuse edema was scored as 3 points, and ankylosing changes in the joints were scored as 4 points. The score was expressed as [total points of all scores in affected mice/number of mice per experimental group] (maximum 16 points). The incidence was calculated as [number of animals with a score of 1 point or greater/number of mice per experimental group].

At Day 82 after the start of the experiment, the animals were sacrificed. The spleen was isolated and weighed, and the spleen weight ratio relative to the body weight was calculated.

The data were expressed as mean  $\pm$  S.E. The tests of significance were conducted by the Mann-Whitney U-test for the mortality rate, joint inflammation score and incidence, and by the Student's t-test for others.

## <Results>

### [I] Endotoxin shock and fatal fulminant hepatitis models (Fig. 1)

The consumption of the Agaro-oligosaccharides during the experimental period, when calculated as agar, was about 20 g/kg/day in the 10% administration group, about 9 g/kg/day in the 5% administration group and about 2 g/kg/day in the 1% administration group.

(1) Deaths occurred from endotoxin shock in 7 of 8 animals in the control group and in 2 of 8 animals in the group receiving 10% Agaro-oligosaccharides, indicating a

significant protective effect.

- (2) The serum TNF- $\alpha$  concentration after LPS administration in the group receiving 10% Agaro-oligosaccharides was suppressed by up to about 70%, but this not significant.
- (3) In the fatal fulminant hepatitis model, the survival was better in the group receiving 10% Agaro-oligosaccharides (4 of 7 animals) compared to the control group (8 of 8 animals).

## [II] Mouse type II collagen-induced arthritis model

[Assessment of Preventive Effect] The consumption of Agaro-oligosaccharides calculated from the mean water consumption during the experimental period was about 4.0 g/kg in the 3% water consumption group and about 0.6 g/kg in the 0.3% water consumption group when expressed as the quantity of agar (Fig. 2).

- ① Change in body weight: The 3% water consumption group showed a transient decrease in the body weight at the start of the experimental water consumption. The weight recovered thereafter, and there was no significant decrease compared to the control group on Day 82, which was the day of sacrifice.
- ② Effect on joint inflammation score and incidence: The control group showed a markedly elevated score from Day 62 to the day of sacrifice, while the 3% water consumption group showed a significant inhibition of this elevation. The 0.3% water consumption group showed no effect. The incidence of joint inflammation in the 3% water consumption group showed a trend towards a decrease but was not statistically significant (Fig. 3).
- ③ Effect on spleen weight: On Day 82, the day of necropsy, the control group showed joint inflammation associated with splenomegaly, while in the 3% water consumption group the spleen showed no enlargement and the weight was within the normal range (Fig. 6).

[Assessment of Therapeutic Effect] The quantity of Agaro-oligosaccharide consumption calculated from the mean water consumption during the period of experiment was about 4.5 g/kg in the 3% water consumption group and about 0.7 g/kg in the 0.3% water consumption group when expressed as the quantity of agar (Fig. 4).

- ① Change in body weight: The 3% water consumption group showed a transient decrease in the body weight with the start of the experimental water consumption. The weight recovered thereafter, and there was no significant decrease compared to the control group on Day 82, which was the day of sacrifice.

- ② Effect on joint inflammation score and incidence: The 3% water consumption group showed a significant inhibition in the score from Day 62 to the day of sacrifice, and incidence of joint inflammation was also significantly decreased on Day 62 (Fig. 5).
- ③ Effect on spleen weight: As with the preventive effect, the 3% water consumption group showed no splenomegaly, and the weight was within the normal range (Fig. 6).

### <Discussion>

The outcome of endotoxin shock and fatal fulminant hepatitis was significantly better in mice provided with water with Agaro-oligosaccharides. In endotoxin-induced fatal shock, it is known that inflammatory cytokines such as TNF- $\alpha$ , IL-1 and NO play important roles<sup>3),4)</sup>, and that PAF and LTs are also involved. The interaction of these effects results in the endotoxin-induced shock state<sup>5)</sup>. Since Agaro-oligosaccharides inhibit NO synthesis *in vitro*, it is thought that, in endotoxin-induced shock, it prevents NO-mediated hypotension and prevents shock-induced deaths<sup>6)</sup>. The inflammatory cytokines are also thought to play an important role in endotoxin-induced liver injury<sup>7)</sup>. Unlike the situation with endotoxin fatal shock, it has been suggested that NO slows the hepatic microcirculation and protects the hepatocyte<sup>8)</sup> and that the administration of NO synthase inhibitors may exacerbate liver injury<sup>9)</sup>. In the present study, unlike other NO synthase inhibitors, the Agaro-oligosaccharides did not exacerbate the endotoxin-induced liver injury (fatal fulminant hepatitis model) but in fact prevented deaths. Chemotherapy in cancer treatment is effective but detrimental to the patient's defense system against infections. Sepsis due to bacterial infection under such a condition may be fatal. The Agaro-oligosaccharides have anti-tumor activity without such adverse effects, and moreover, through its inhibition of NO synthesis and inhibition of TNF- $\alpha$  production, may improve mortality in sepsis.

Rheumatoid arthritis is a type of autoimmune disease primarily manifesting as joint lesions, and number of patients with this disease is increasing in this aging society. In type II collagen-induced arthritis in the mouse, which is a widely used experimental model of rheumatoid arthritis, consumption of water containing Agaro-oligosaccharides had not only a preventive effect but also a therapeutic effect. In rheumatoid arthritis, as in endotoxin shock, it is also known that inflammatory cytokines such as TNF- $\alpha$ , IL-1, IL-6<sup>10),11),12)</sup>, NO<sup>13)</sup> and active oxygen are intimately involved in the pathogenesis of the disease. In this study, the Agaro-oligosaccharides improved the joint inflammation score and incidence of joint inflammation and also clearly inhibited the proliferative reaction of splenocytes to type II collagen stimulation.

In this model, it is known that the anti-collagen antibody titer in the blood increases<sup>14),15)</sup>, and in the mice with joint inflammation, the splenomegaly is due to activation of antibody-producing cells. It is unknown whether the inhibition of splenomegaly by the Agaro-oligosaccharides is the result of or a cause of the inhibition of joint inflammation. In addition, in this model, the involvement of cell-mediated immunity has also been suggested to be important<sup>16)</sup>. The involvement of both humoral immunity and cell-mediated immunity in the chronic nature and severity of the disease suggests similarity of this model to the rheumatoid arthritis in humans<sup>17)</sup>. In the future studies, we anticipate that the utility of Agaro-oligosaccharides will be demonstrated in human rheumatoid arthritis and consider that a detailed mechanism of action must be elucidated.

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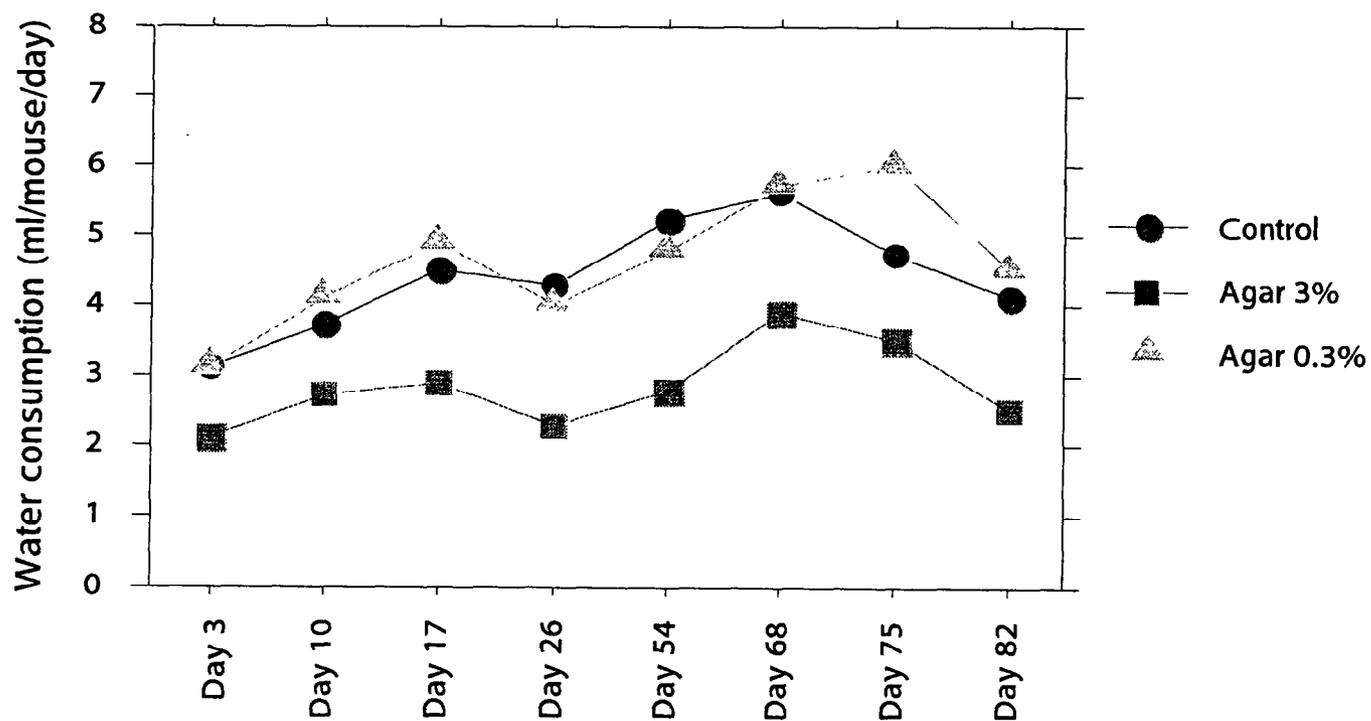
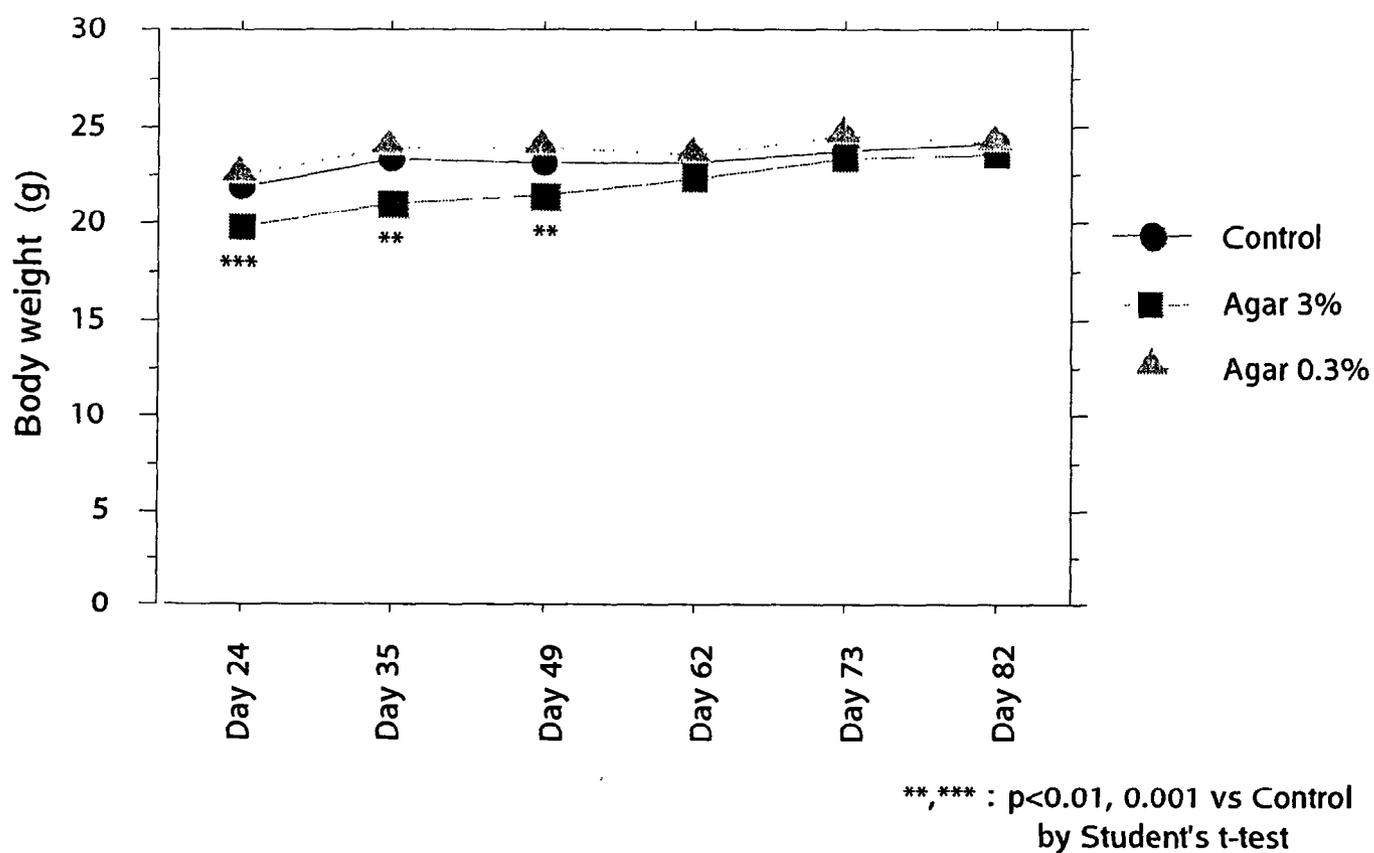
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Group	(1) Lethality death /total(%)		(2) TNF- $\alpha$ ( $\mu$ g/ml)	(n)	(3) Lethality death/total (%)	
Control	7/8	(88)	10.4 $\pm$ 1.6	(4)	8/8	(100)
Agaro- oligosaccharide 10%	2/8*	(25)	7.4 $\pm$ 0.8	(4)	4/7*	(57)
5%	6/8	(75)	12.8 $\pm$ 1.6	(4)	7/8	(88)
1%	8/8	(100)	11.3 $\pm$ 1.0	(4)	8/8	(100)

\* : p<0.05 vs Control by Mann-Whitney U-test

Fig.1 Improvement in mortality and inhibition of TNF-  $\alpha$  production by Agaro-oligosaccharides in endotoxin shock and fatal fulminant hepatitis models



**Fig.2 Preventive effect of Agaro-oligosaccharides in the type II collagen-induced arthritis model in mice**

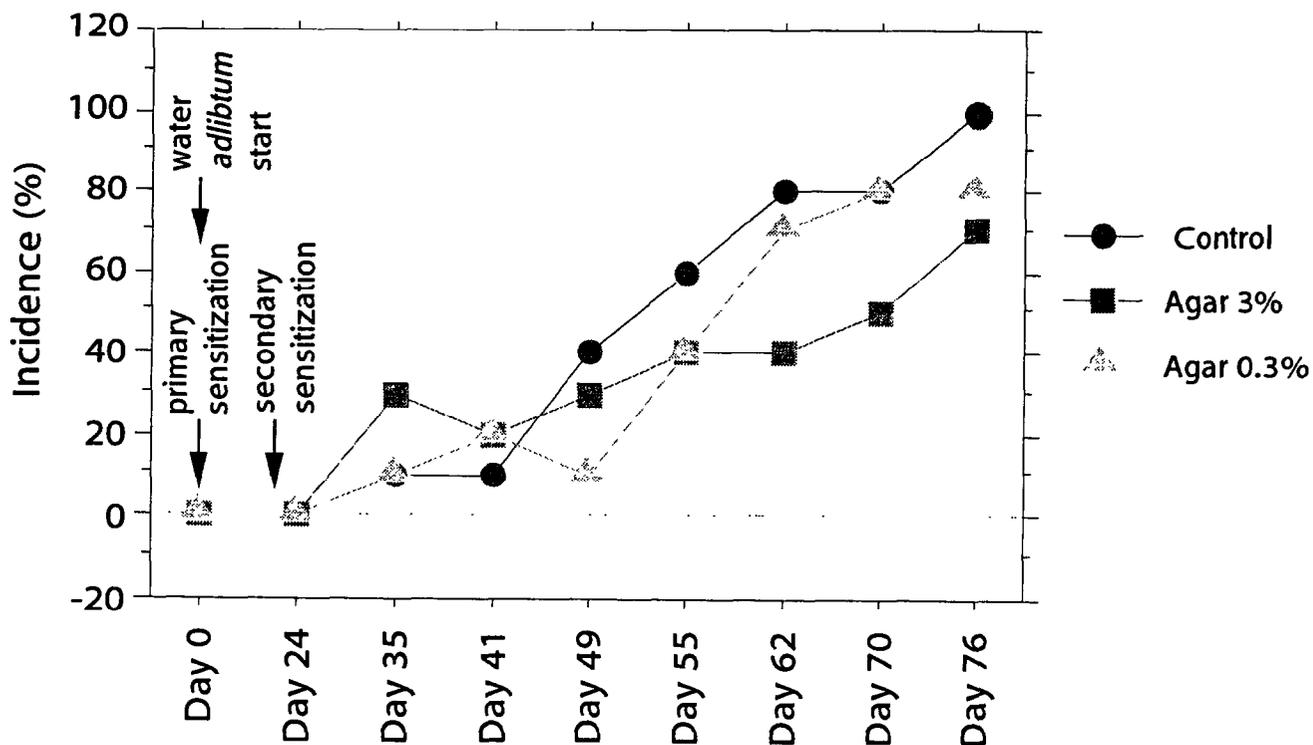
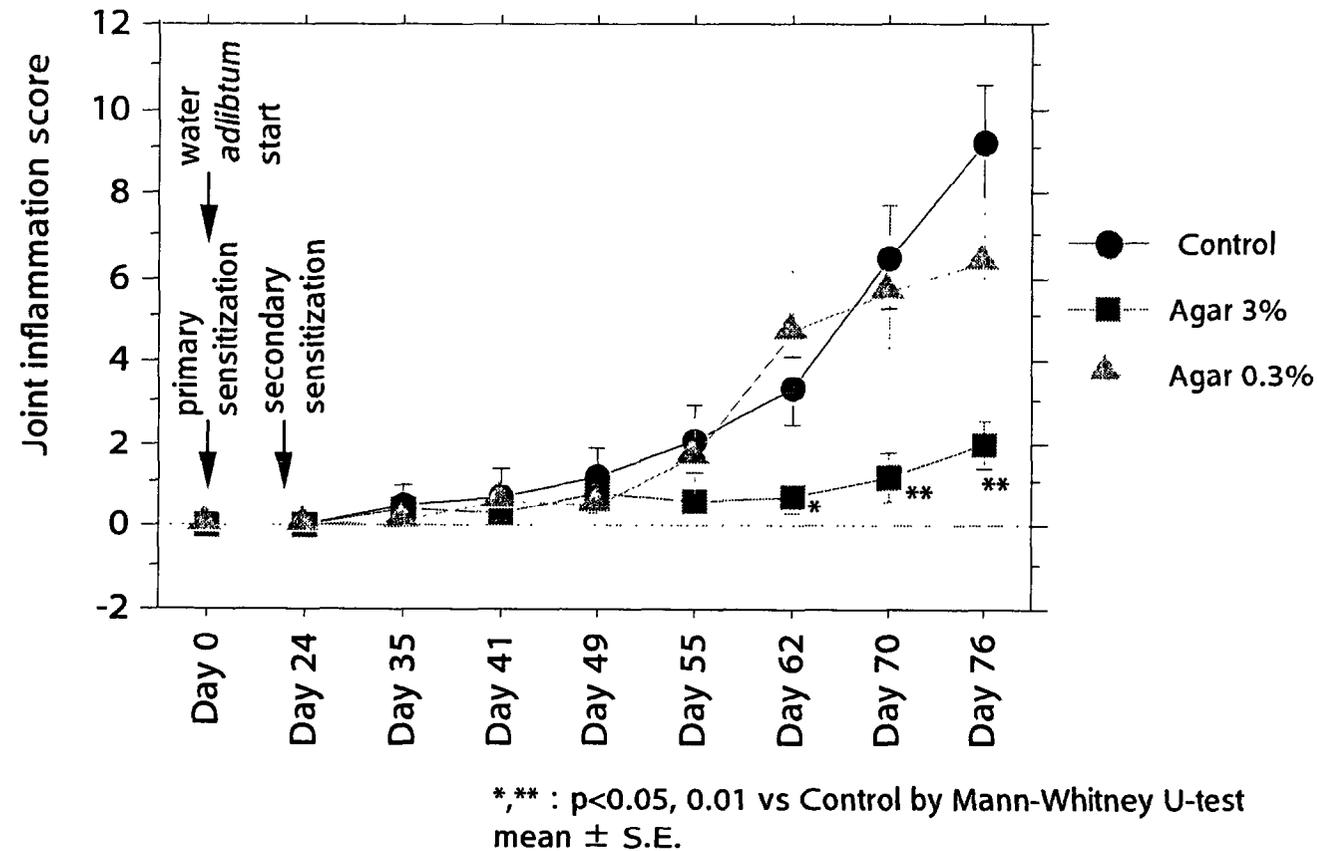


Fig.3 Preventive effect of Agaro-oligosaccharides in the type II collagen-induced arthritis model in mice

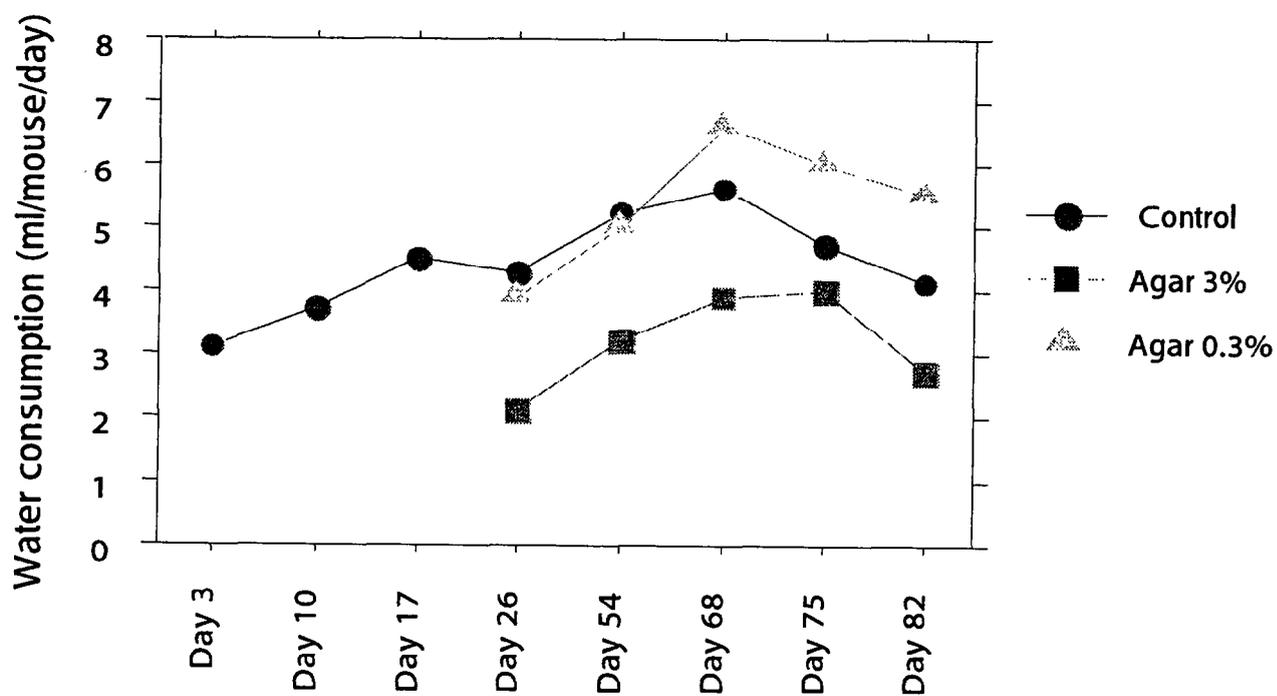
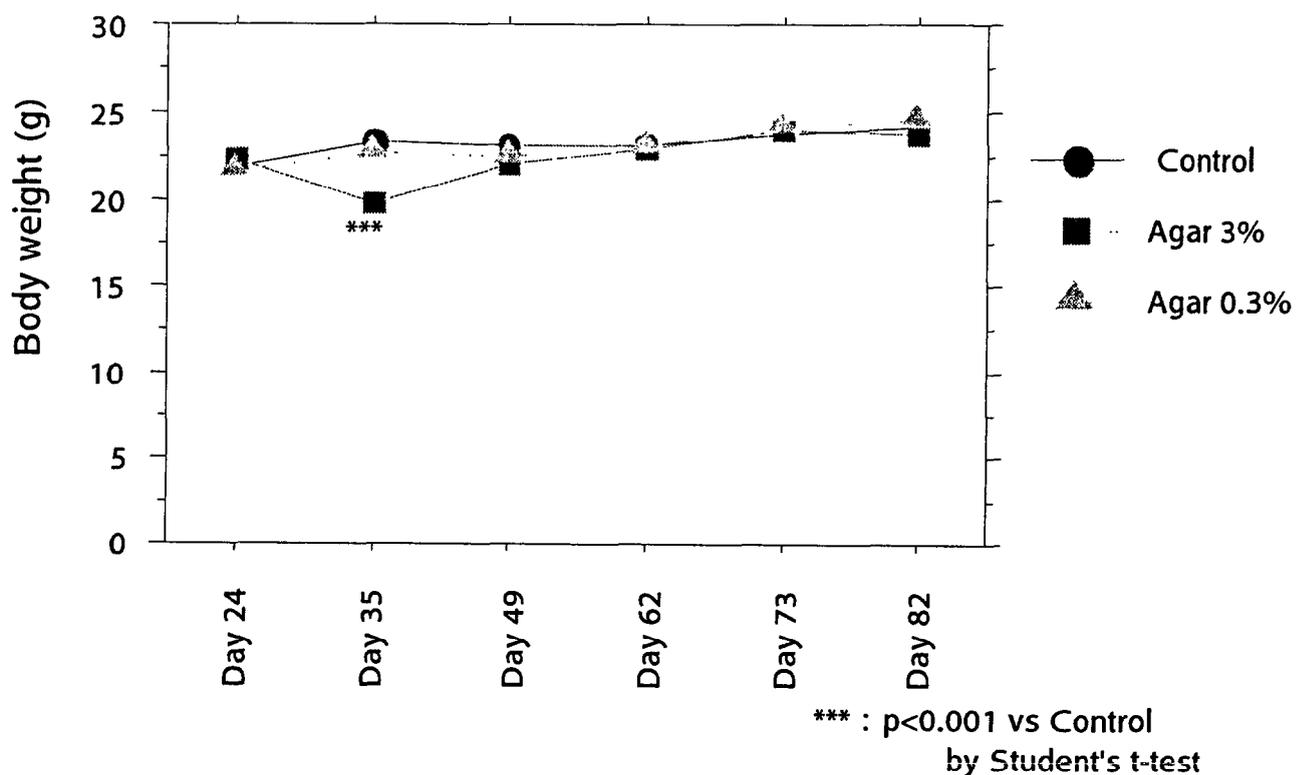
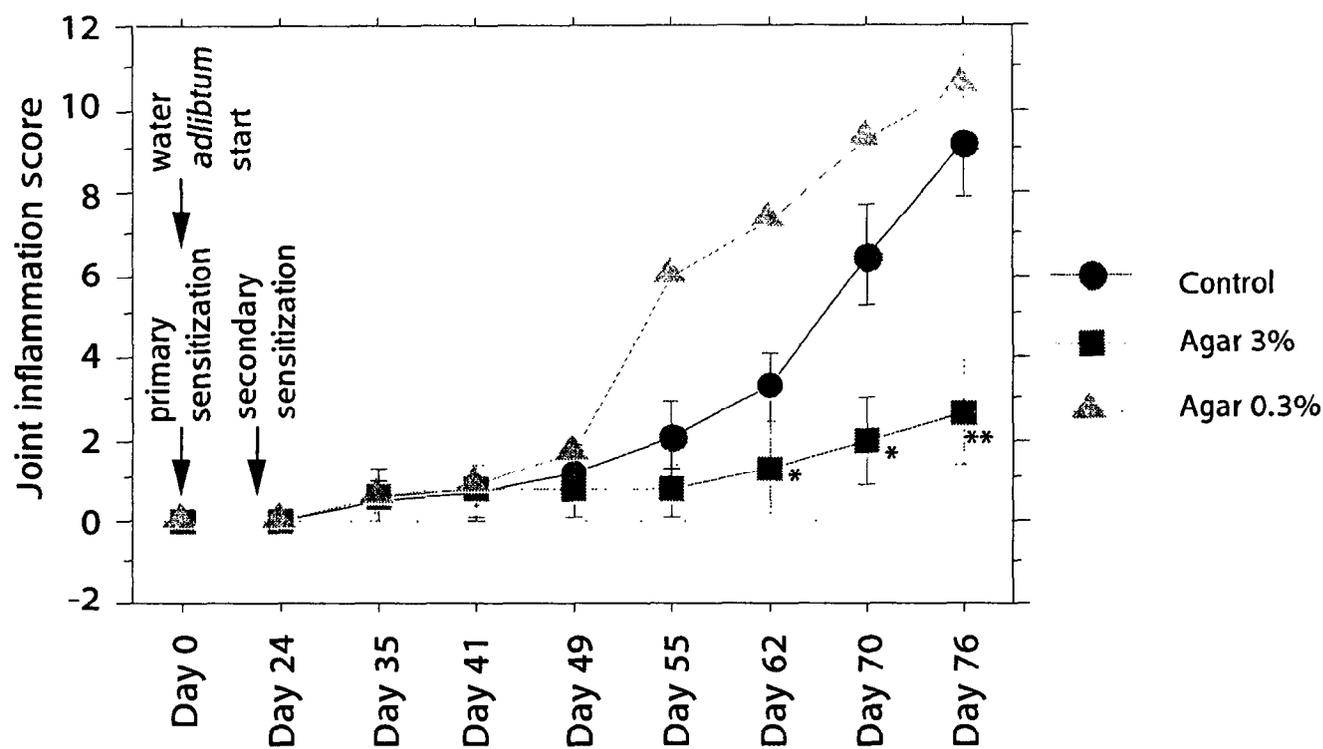
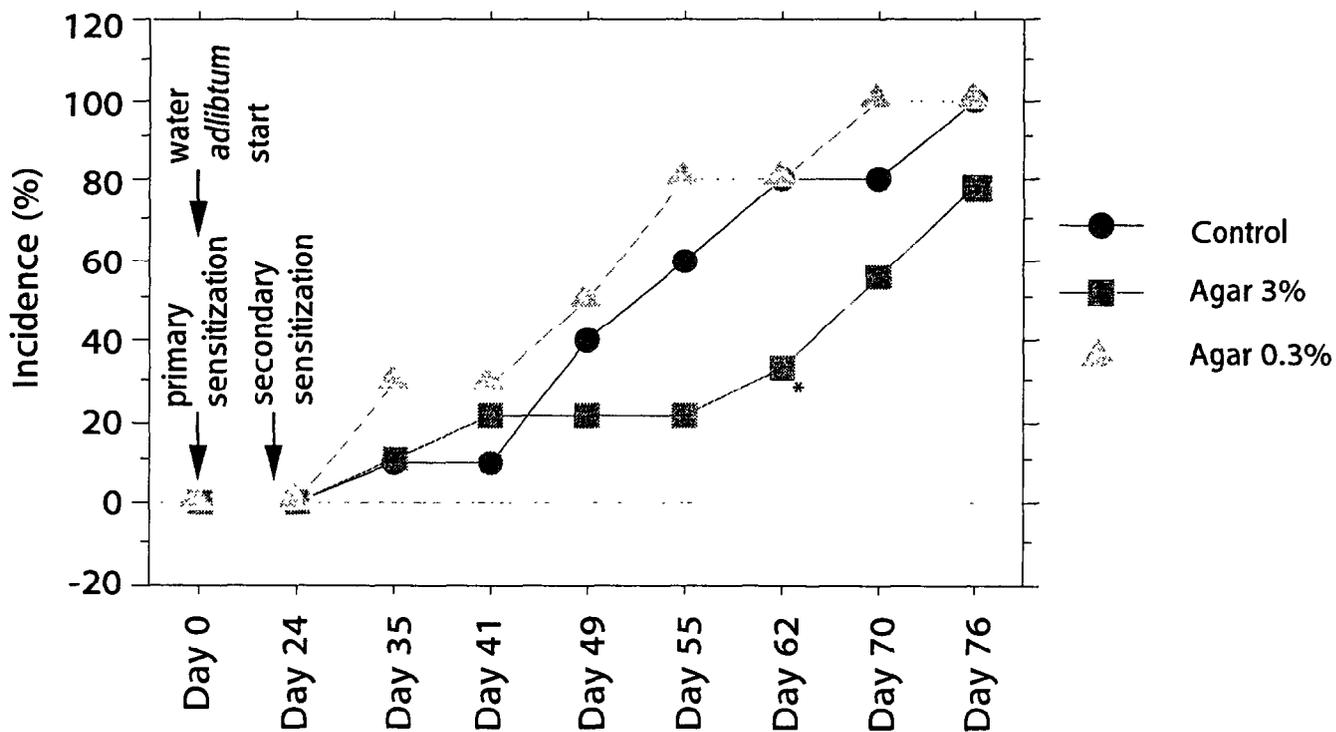


Fig.4 Therapeutic effect of Agaro-oligosaccharides in the type II collagen-induced arthritis model in mice



\*,\*\* : p<0.05, 0.01 vs Control by Mann-Whitney U-test mean ± S.E.



\* : p<0.05 vs Control by Mann-Whitney U-test

Fig.5 Therapeutic effect of Agaro-oligosaccharides in the type II collagen-induced arthritic model in mice

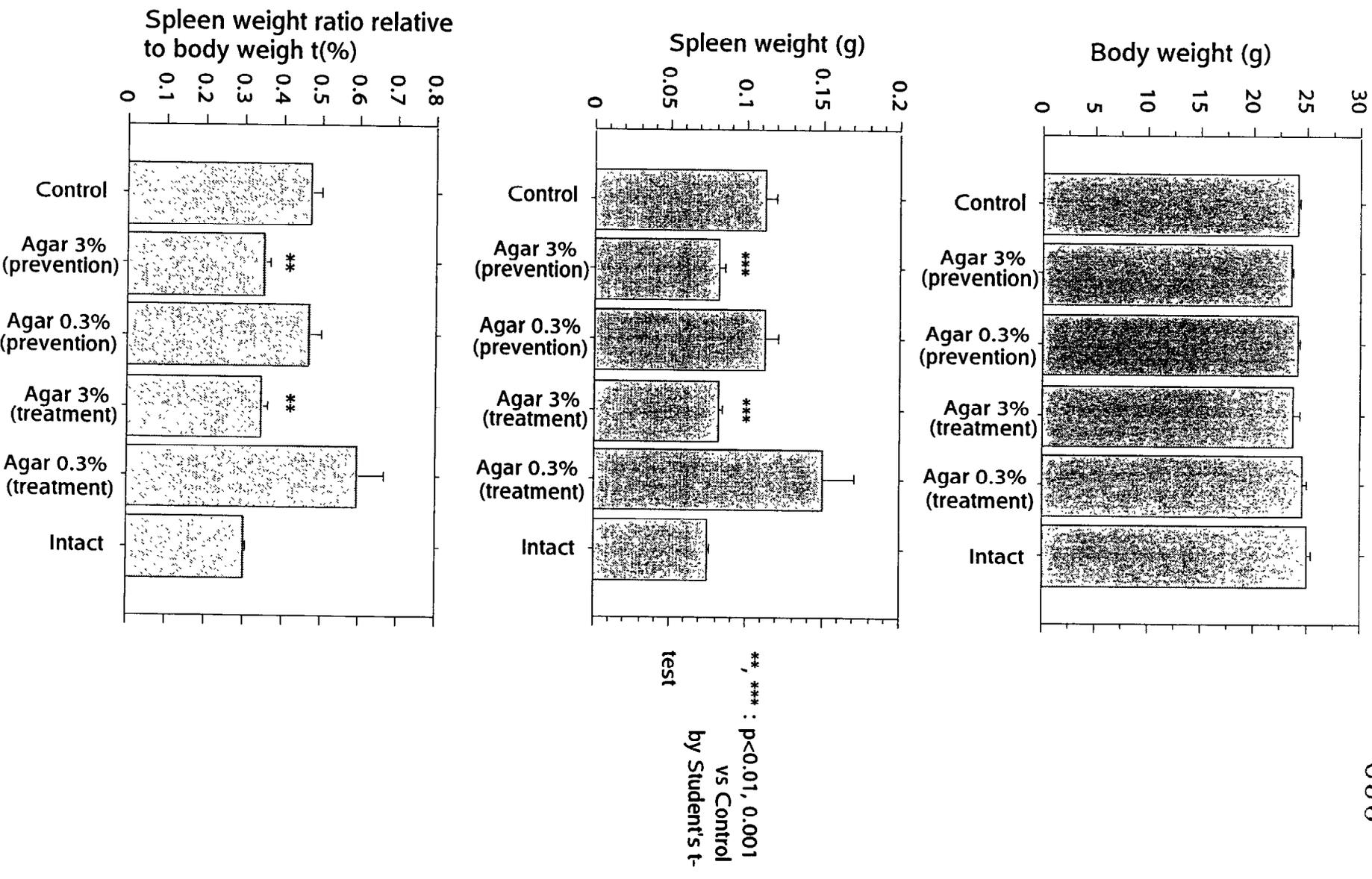


Fig.6 Effect of Agaro-oligosaccharides in the type II collaen-induced arthritis model in mice

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## Anti-Inflammatory Activities of Agaro-Oligosaccharides Have Beneficial Effects to Arthritis

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### Introduction

Many cytokines and inflammatory mediators are expressed in Rheumatoid arthritis (RA). In the pathogenesis of this disease, neutrophils and macrophages are activated, resulting in the release of activated oxygen and proteases and the destruction of cartilage and tissue. Therefore, as a therapeutic strategy, it is considered important to inhibit the release of activated oxygen and proteases and inhibit the migration and infiltration of neutrophils and macrophages by suppressing inflammatory mediators and cytokines.

Anti-inflammatory agents have long been used to treat RA. The synovial tissue in RA patients expresses cyclooxygenase-2 (COX-2), which is inducible by mitogenic stimuli<sup>1)</sup>, so that Prostaglandins are synthesized from Arachidonic acid derived from cell membrane phospholipid. The Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), which is specifically produced by COX-2, increases the blood flow and potentiates the edema and white cell infiltration. Anti-inflammatory agents inhibit the activity of COX-2 and the synthesis of Prostaglandins, thus suppressing inflammation.

It has been also known that the serum Nitric oxide (NO) level is elevated in RA patients<sup>2,3)</sup>. The cellular source of the NO has not yet been clearly identified, but the NO is known to be increased in the synovium<sup>2,3)</sup> and thus may be synthesized by activated macrophages or neutrophils. At the sites of inflammation, the activated macrophages show persistently elevated expression of inducible NO synthase (iNOS), resulting in the production of large amounts of NO. The NO reacts with the activated oxygen O<sub>2</sub><sup>-</sup> (super anion) produced by the activated neutrophils present at the inflammatory sites to produce the highly toxic ONOO<sup>-</sup> (peroxynitrite)<sup>4)</sup>. Therefore, the elevated NO is thought to lead to further progression of the disease. Although there are no drugs under development aimed at the inhibition of NO production, this is a clearly a reasonable therapeutic target.

Furthermore, pro-inflammatory cytokines, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, etc., are known to be involved in the pathogenesis of RA<sup>5)</sup>. Recently, new RA drugs that target such

pro-inflammatory cytokines have been developed, for example, the anti-TNF- $\alpha$  antibody in clinical use that inhibits TNF- $\alpha$  and thus suppress the release and migration of white blood cells. In addition, the inhibition of TNF- $\alpha$  has been reported to suppress the production of IL-1 and IL-6<sup>6</sup>).

Thus, multiple factors are thought to be involved in the pathogenesis of RA, and several therapeutic approaches exist.

Agar is contained in large amounts in seaweeds such as the Gelidiales group of marine algae and Japanese nori and has long been a component of the Japanese diet. Most of the Agarose contained in the agar is a polysaccharide in which  $\beta$ -D-galactose and  $\alpha$ -3,6-anhydro-L-galactose are combined each other at the 3 position and at the 4 position, respectively. Acid hydrolysis selectively breaks the  $\alpha$  bond to produce Agaro-oligosaccharides, which are disaccharides with 3,6-anhydro-L-galactose at the reducing end<sup>7,8,9</sup>). We have previously demonstrated that Agaro-oligosaccharides induce heme oxygenase-1 (HO-1) and inhibit the NO synthesis by activated macrophages<sup>10</sup>). Heme oxygenase is an enzyme that degrades heme into CO (carbon monoxide), iron and biliverdin and is known to exist as the inducible form HO-1 and the constitutive forms HO-2 and HO-3<sup>11,12,13</sup>). Recently, it has been shown that the CO synthesized by Heme oxygenase has anti-inflammatory effects and inhibits the TNF- $\alpha$  and IL-1  $\beta$  synthesized by LPS-activated macrophages<sup>14</sup>). Thus, Agaro-oligosaccharides may be expected to exert anti-inflammatory effects by inducing Heme oxygenase-1, thereby increasing CO production and inhibiting the synthesis of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1  $\beta$  and IL-6. We now report that the Agaro-oligosaccharides induce Heme oxygenase-1, inhibit the synthesis of NO and activate oxygen, inhibit the synthesis of the pro-inflammatory mediator PGE<sub>2</sub>, and inhibit the production of pro-inflammatory cytokines TNF- $\alpha$ , IL-1  $\beta$  and IL-6, suggesting that Agaro-oligosaccharides may have preventive and therapeutic effects in RA through a variety of mechanisms.

## Materials and methods

### Reagents

Lipopolysaccharide (LPS, *Escherichia coli* 055:B5) and neo-Agarobiose were obtained from Sigma. Recombinant mouse Interferon- $\gamma$  (IFN- $\gamma$ ) was obtained from Genzyme. Other reagents were obtained from Nacalai Tesque, unless otherwise specified.

### **Preparation of Agaro-oligosaccharides**

Agar was resuspended in 0.1N HCl to 10% (w/v) and heated at 100°C for 15 min. After centrifuging the sample to remove the insolubles, the Agaro-oligosaccharide solution was subjected to gel filtration chromatography in Toyopearl HW-40C (45 mm × 100 cm, Toso) to obtain Agaro-oligosaccharides of various lengths. The gel filtration chromatography was conducted at the rate of 1 mL/min and pure water was used as the carrier. The various fractions were analyzed by thin layer chromatography (TLC) using silica gel 60 F<sub>254</sub> and butanol : ethanol : water (5 : 3 : 3) as the solvent system. The TLC analysis results indicated that various Agaro-oligosaccharide fractions were obtained, so that Agarobiose, Agarotetraose and Agaro-hexaose were prepared.

### **Cell culture of RAW264.7**

The mouse macrophage cell line RAW264.7 (ATCC TIB71) was obtained from Dainippon Pharmaceutical Co., Ltd. The cells were cultured in Dulbecco's modified Eagle's medium (BioWhittaker) containing 10% fetal bovine serum (JRH Biosciences) and antibiotics (50 units/mL penicillin, 50 µg/mL streptomycin, Gibco BRL). The cells were passaged every 3 days. For the evaluation of NO synthesis, the cells were suspended in culture medium to give  $4 \times 10^5$  cells/mL and placed in 48-well plates at 0.5 mL. For evaluation of HO-1 induction, the cells were suspended in culture medium to give  $3 \times 10^5$  cells/mL and placed in 6-well plates at 5 mL. After overnight culture, fresh medium was added, and the assays were conducted.

### **Evaluation of HO-1 induction**

To the RAW264.7 cells, various concentrations of Agaro-oligosaccharide solutions were added and cultured for 12 hr. At the end of the culture period, the cells were collected, washed in PBS, resuspended in lysis buffer (0.1% Triton X-100, 10 mM EDTA-2Na, 1 mM PMSF, 0.2 mM leupeptin, 0.05 mM pepstatin A in 100 mM Tris HCl, pH 7.4), and subjected to one cycle of freeze-thaw to prepare the cell extract. This was then centrifuged at 4°C, 10,000 rpm, for 20 min, and the supernatant was collected. The protein concentration in the samples was measured using the Micro BCA protein assay reagent (Pierce Chemical Company). Cell extract containing 10 µg of the protein was mixed with the Laemmli loading buffer, heated at 100°C for 5 min, and applied to 12.5% polyacrylamide gel. After electrophoresis, the proteins were transferred to PVDF membrane (Millipore Japan). The signal was detected by chemiluminescence (Renaissance, NEN Life Science Products) using anti-HO-1 polyclonal antibody (Santa Cruz Biotechnology), with anti- $\alpha$  tubulin monoclonal antibody (Calbiochem) as the

internal control.

### **Evaluation of NO synthesis**

Agarobiose was added to RAW264.7 cells at various concentrations and cultured for 5 hr. LPS was then added to a final concentration of 1  $\mu\text{g}/\text{mL}$  and IFN- $\alpha$  to final concentration of 10 U/mL and cultured for 16 hr. After the incubation period, the culture medium was assayed for nitrite formed as a result of NO hydrolysis as an index of NO synthesized. 100  $\mu\text{L}$  of the culture supernatant was treated with 100  $\mu\text{L}$  of the 4% Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylene diamine in 5% o-phosphoric acid, Sigma) and measured after 15 min in a plate reader at 540 nm absorbance.

### **Isolation and storage of human peripheral blood mononuclear cells (PBMC)**

400 mL of blood was obtained from human healthy donors. The blood was diluted 2 fold in PBS(-), overlaid on Ficoll-paque (Pharmacia) and centrifuged for 20 min at  $500 \times g$ . The peripheral blood mononuclear cells (PBMC) at the interphase were collected with a pipette and washed in RPMI1640 medium (BioWhittaker). The PBMC collected was resuspended in the freezing solution of 90% FCS (JRH Biosciences)/10% dimethyl sulfoxide, and stored in liquid nitrogen. At the time of the experiment, the PBMC stored was rapidly thawed in a 37°C water bath, washed in RPMI1640 medium containing 10  $\mu\text{g}/\text{mL}$  DNase (Calbiochem), and cell viability tested by the trypan blue dye exclusion method.

### **Isolation of human PBMC derived monocytes**

The PBMC was suspended at  $2 \times 10^6$  cells/mL in RPMI 1640 medium containing 5% human AB serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine (all from BioWhittaker), 10 mM HEPES and antibiotics (50 units/mL penicillin, 50  $\mu\text{g}/\text{mL}$  streptomycin, Gibco BRL) (5H RPMI medium), plated in 24-well plates at 1 mL/well, and incubated for 1.5 hr. At the end of 1.5 hr, the non-adherent cells were removed, and each well was washed with RPMI 1640, and then 1 mL of 5H RPMI medium added to obtain human monocytes.

### **Evaluation of production of PGE<sub>2</sub> and various cytokines**

Agarobiose was added at various concentrations to the human monocytes and cultured for 5 hr. LPS was then added to the final concentration of 1 ng/mL, and culture continued for 16 hr for PGE<sub>2</sub> measurements or for 4 hr for measurements of

TNF- $\alpha$ , IL-1 $\beta$  and IL-6. The culture supernatants were collected and the PGE<sub>2</sub>, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 contents determined using ELISA kits (PGE<sub>2</sub> (Neogen), TNF- $\alpha$  (Endogen), and IL-1 $\beta$  and IL-6 (Genzyme Techne)).

## Results

### Induction of Heme oxygenase-1 by Agaro-oligosaccharide

Induction of Heme oxygenase-1 by Agaro-oligosaccharide was studied in the mouse macrophage cell line RAW264.7 cells. As indicated in Figure 1, with medium alone, RAW264.7 cells at 12 hr later showed no detectable levels of HO-1. In contrast, the addition of Agaro-oligosaccharides resulted in a dramatic induction of HO-1. This HO-1 induction was seen with all Agaro-oligosaccharides, Agarobiose, Agarotetraose and Agarohexaose, and the expression level increased in a dose dependent manner. On the other hand, neo-Agarobiose, which has a galactose at the reducing end, did not induce HO-1 expression. The HO-1 induction by Agaro-oligosaccharides was determined to be at the transcriptional level by RT-PCR. It was also determined that HO-1 induction occurred at the transcriptional level in human PBMC derived monocytes (data not shown).

### Studies on inhibition of NO production by Agarobiose

Using the RAW264.7 cells we studied the effect of Agarobiose on nitrite accumulation after LPS or IFN- $\gamma$  stimulation. As an index of NO synthesis by cells, we measured the accumulation of nitrite produced by hydrolysis of NO by water present in the culture supernatant using the Griess reagent. Using this system, we determined that even a high concentration of Agarobiose (1 mM) had no effect on the reaction of nitrite with the Griess reagent (data not shown). Nitrite production by RAW264.7 was dependent on the activation state. Activation by LPS or IFN- $\gamma$  resulted in a 90-fold increase in the nitrite (about 31  $\mu$ M) compared to the non-stimulated state (about 0.37  $\mu$ M). In contrast, the addition of Agarobiose prior to the stimulation resulted in a dose-dependent inhibition of NO synthesis, with about 50% inhibition at 100  $\mu$ M of Agarobiose (Figure 2). Similar results were obtained with Agarotetraose and Agarohexaose, while neo-Agarobiose gave no inhibition (data not shown). In this experimental system, Agaro-oligosaccharides had no cellular toxicity even at high concentrations as determined by the MTT assay (data not shown).

### **Assessment on inhibition of PGE<sub>2</sub> and pro-inflammatory cytokine production by agarobiose**

Effect of Agarobiose on LPS-stimulated production of PGE<sub>2</sub> and pro-inflammatory cytokines was studied in human PBMC derived monocytes. The assays were conducted using commercially available ELISA kits; with all kits it was determined that Agarobiose even at high concentrations (1 mM) had no effect on the reaction system (data not shown). The production of PGE<sub>2</sub> and various pro-inflammatory cytokines by human monocytes requires an activated state, and LPS stimulation results in an increase in PGE<sub>2</sub> by two fold (about 47 ng/mL) compared to the unstimulated state (about 23 ng/mL), an increase in TNF- $\alpha$  by about 60 fold (to about 3000 pg/mL) compared to the unstimulated state, an increase in IL-1 $\beta$  by about 6 fold (to about 240 pg/mL) compared to the unstimulated state (about 40 pg/mL), and increase in IL-6 by about 4 fold (to about 1200 pg/mL) compared to the unstimulated state (about 300 pg/mL). In contract, the addition of Agarobiose prior to stimulation resulted in dose-dependent inhibition of the production of PGE<sub>2</sub> and pro-inflammatory cytokines. The addition of 50  $\mu$ M Agarobiose resulted in the inhibition of production of PGE<sub>2</sub> by about 75%, TNF-  $\alpha$  by about 57%, IL-1  $\beta$  by essentially 100%, and IL-6 by about 50% (Figure 3 (a) PGE<sub>2</sub>; 3(b) TNF-  $\alpha$  ; 3(c) IL-1  $\beta$  ; 3(d) IL-6). In this experimental system, it was also confirmed that Agarobiose at high concentrations had no cellular toxicity by the MTT assay (data not shown).

### **Discussion**

In this study, it was demonstrated that Agaro-oligosaccharides, oligosaccharides derived from a dietary fiber, inhibit the production of NO, PGE<sub>2</sub> and various pro-inflammatory cytokines by macrophages.

It has previously been thought that dietary fiber such as agar has no notable physiological activity and that any cancer preventive property may be attributable to physical effects such as improvement in stool transit. There have essentially been no studies conducted on physiological activity of dietary fiber and oligosaccharides. Specifically, inhibition of activated macrophages and anti-inflammatory effects by dietary fiber and oligosaccharides is to the best of our knowledge previously unreported.

Agaro-oligosaccharides, which are structural oligosaccharides derived agar, induced Heme oxygenase-1 in the mouse macrophage cell line RAW264.7 in a dose dependent manner. This induction was seen with Agaro-oligosaccharides having the 3,6-anhydro-L-galactose at the reducing end, such as Agarobiose, Agarotetraose and Agarohexaose, while neo-Agarobiose with D-galactose at the reducing end was inactive.

In the studies of inhibition of NO production in the same RAW264.7 cells, Agaro-oligosaccharides inhibited NO synthesis, but neo-Agarobiose had no activity. Therefore, the physiological activity of the Agaro-oligosaccharides appears to require 3,6-anhydro-L-galactose at the reducing end and that this is responsible for the induction of HO-1 and inhibition of NO production.

When HO-1 is induced, heme is degraded, releasing biliverdin, iron and CO<sup>11)</sup>. The biliverdin is metabolized to bilirubin through the effects of biliverdin reductase. This bilirubin has attracted attention as a physiological compound with antioxidant properties involved in the elimination of activated oxygen species and prevention of lipid peroxidation<sup>15,16,17)</sup>. Thus, at inflammatory sites it may remove activated oxygen species and prevent worsening of the lesions. Therefore, HO-1 induction by Agaro-oligosaccharide may inhibit the production of NO by macrophages and the production of activated oxygen by neutrophils that have infiltrated RA lesions and inhibit the injury directly. Recently, Choi et al., reported that the CO produced through the action of HO-1 inhibits the production of pro-inflammatory cytokines produced by activated macrophages such as TNF- $\alpha$ , IL-1 $\beta$ , MIP-1 $\beta$  and MIP-2<sup>14)</sup>. Various cytokines have been implicated in RA. The Agaro-oligosaccharides induce HO-1, and the CO thus produced may inhibit the pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6, which have been therapeutic targets. In fact, Agarobiose inhibited production of these cytokines induced by LPS activation of human monocytes. This inhibitory effect is clearly observed when the Agarobiose is added to the monocytes prior to LPS stimulation. Therefore, it was suggested that the Agarobiose induces some sort of factor, resulting in the inhibition of the production of pro-inflammatory cytokines. Whether the Agarobiose directly inhibits all of these pro-inflammatory cytokines is currently unclear. However, since the effect is markedly seen when added prior to stimulation, the mechanisms of inhibition may be similar. Therefore, by adding Agarobiose prior to stimulation, it was suggested that HO-1 is sufficiently induced and CO is produced, thereby suppressing the pro-inflammatory cytokines.

Furthermore, Agarobiose inhibits the PGE<sub>2</sub> production by LPS-stimulated human monocytes. Anti-inflammatory agents such as aspirin have long been known to inhibit COX, which is a key enzyme in prostaglandin synthesis. COX exists as 2 isozymes, COX-1, which is expressed constitutively, and COX-2, which is induced at sites of inflammation by mitogenic stimuli<sup>18,19)</sup>. Conventional non-steroidal anti-inflammatory agents (NSAIDs) inhibit both of these enzymes, and adverse effects are attributable to the inhibition of COX-1, which plays an important role in the protection of the gastric mucosa. Recently developed COX-2 selective NSAIDs inhibit only COX-2.

Agarobiose has no effect on PGE<sub>2</sub> production produced by COX-1 (data not shown), and its inhibitory effect involves a cascade that specifically includes COX-2. It has been shown that this is not due to COX-2 enzyme inhibition, but it has not been determined what step is inhibited in the PGE<sub>2</sub> production. PGE<sub>2</sub> synthase, which is downstream of COX-2, has been recently identified and cloned<sup>20</sup>. Since there have been no reports on the relationship between PGE<sub>2</sub> synthase and CO, further studies are needed to assess the possibility that Agarobiose inhibits the PGE<sub>2</sub> synthase.

Antibody therapeutic agents such as the anti-TNF antibody and anti-inflammatory agents such as NSAIDs are used in the treatment of RA. In the current study, we obtained data suggesting that Agaro-oligosaccharides, which are oligosaccharides derived from dietary fiber, may have preventive and therapeutic effects in RA. Three sites of action are thought to be possible in the prevention and treatment of RA by Agaro-oligosaccharides (Figure 4). Agaro-oligosaccharides induce HO-1, resulting in the synthesis of CO and bilirubin. This may cause (1) the inhibition of NO production by macrophages activated at the sites of inflammation, while the bilirubin may promote the elimination of activated oxygen, to inhibit tissue injury directly; (2) Selective inhibition of the COX-2 cascade in the PGE<sub>2</sub> production in activated macrophages, resulting in anti-inflammatory effects with no adverse effects; (3) inhibition by CO of the synthesis of pro-inflammatory cytokines by activated macrophages, so that induction of inflammation inhibited, resulting in therapeutic benefit.

Therefore, Agaro-oligosaccharides, which are oligosaccharides derived from agar, a dietary fiber used in Japan since ancient times, may be a previously unknown, new type of food product that may be useful in the prevention or treatment of RA at multiple sites of action.

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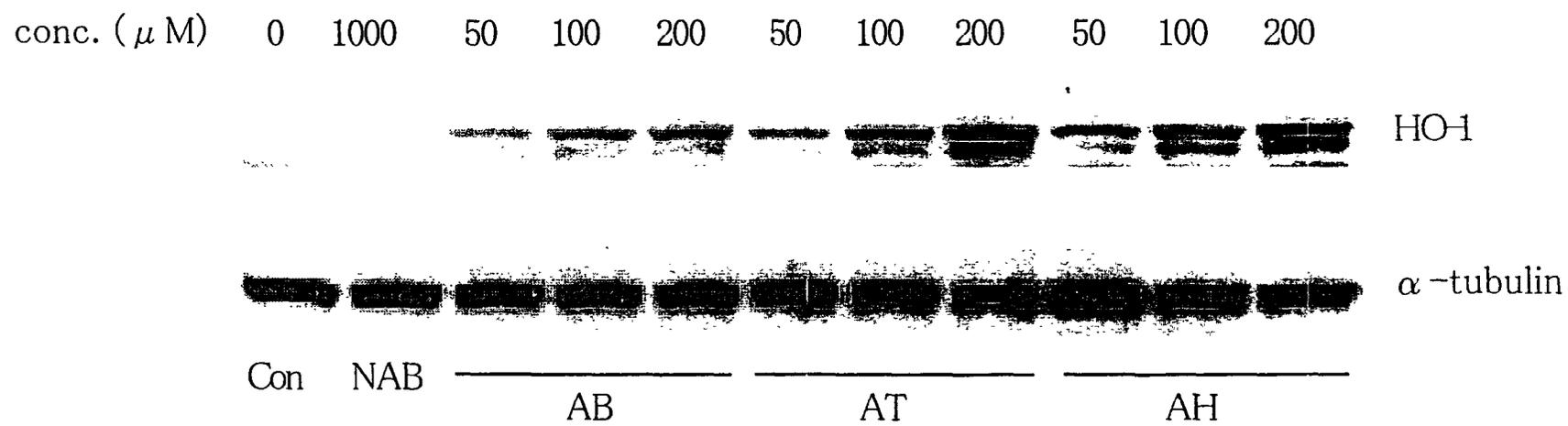


Fig.1 Effects of various oligosaccharides on the levels of HO-1 and  $\alpha$ -tubulin protein

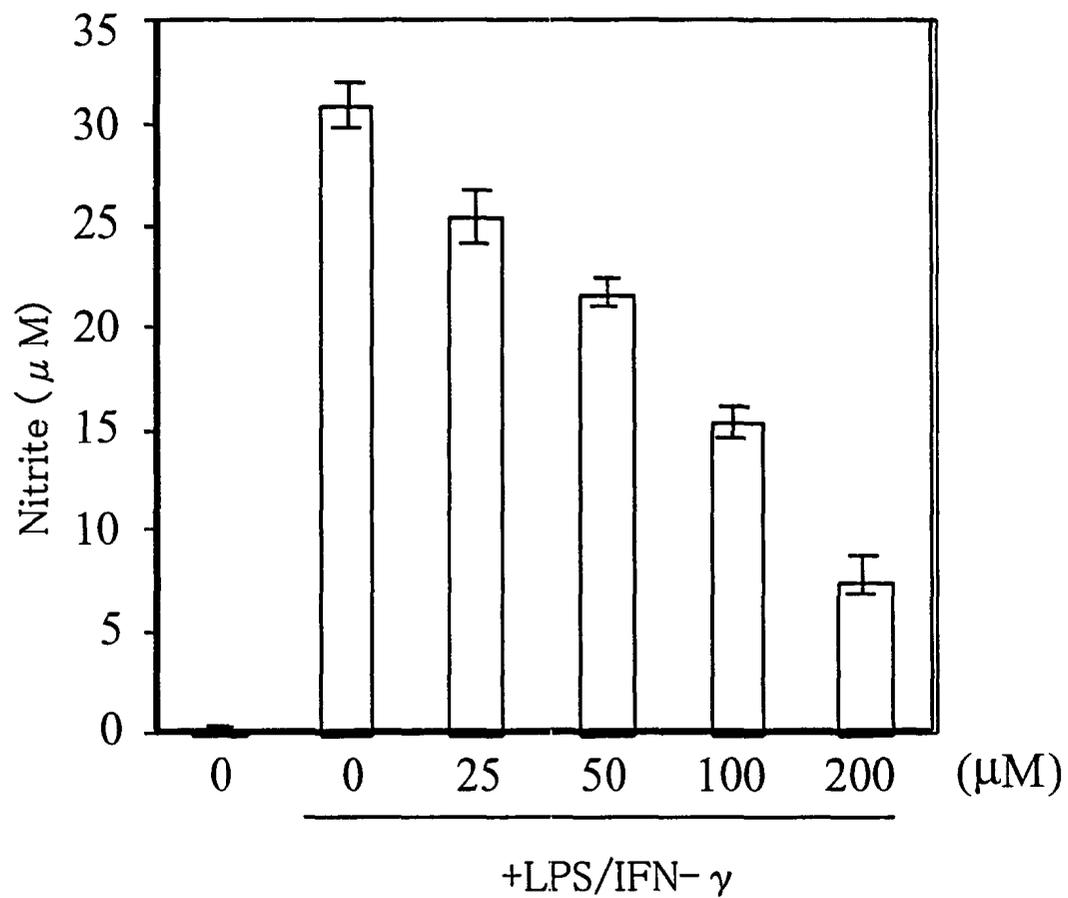


Fig.2 Effects of agarobiose on LPS plus IFN- $\gamma$  induced nitric production in RAW264.7 macrophages

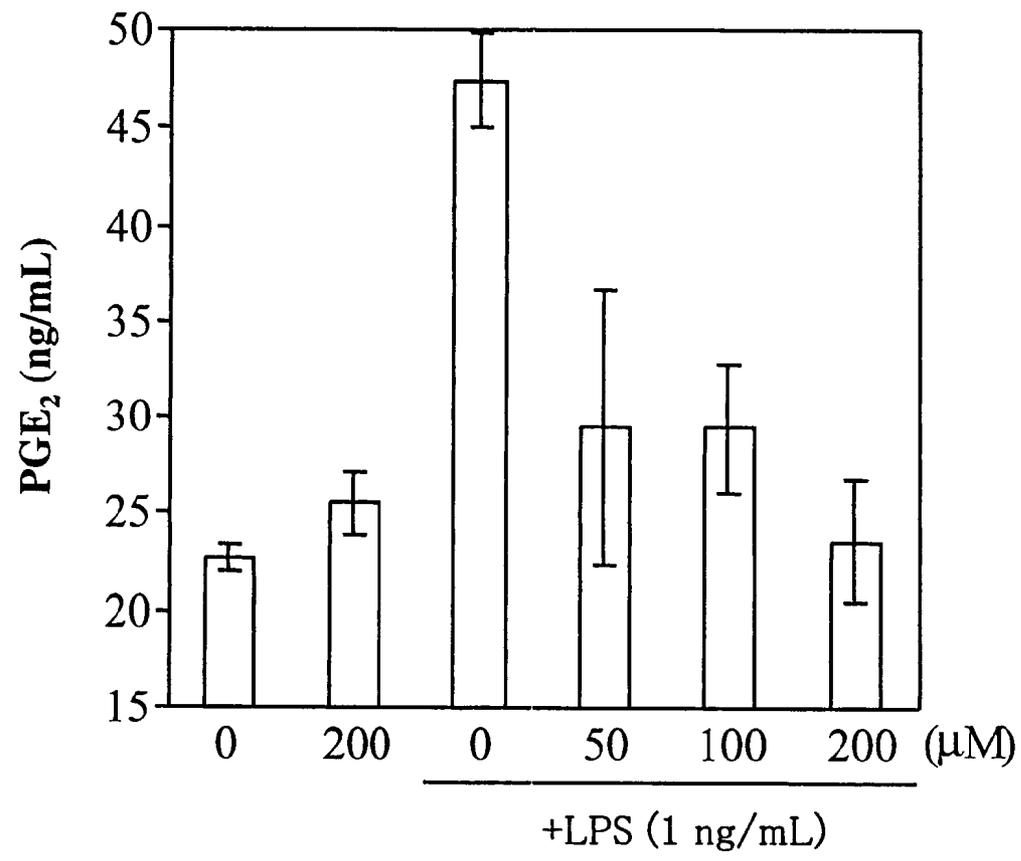


Fig.3(a) Effects of agrobiose on LPS-induced PGE<sub>2</sub>

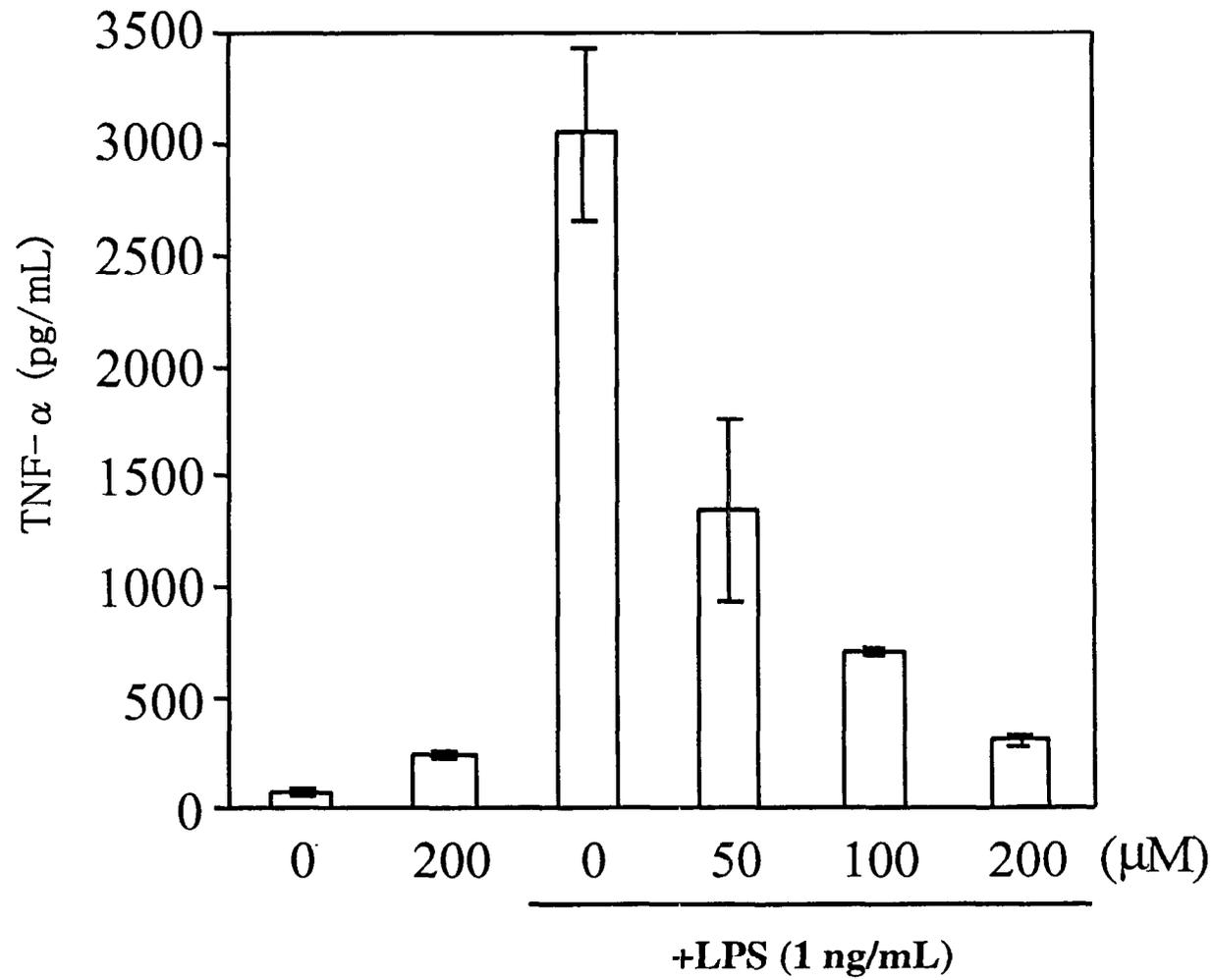


Fig.3(b) Effects of agarobiose on LPS-induced TNF- $\alpha$

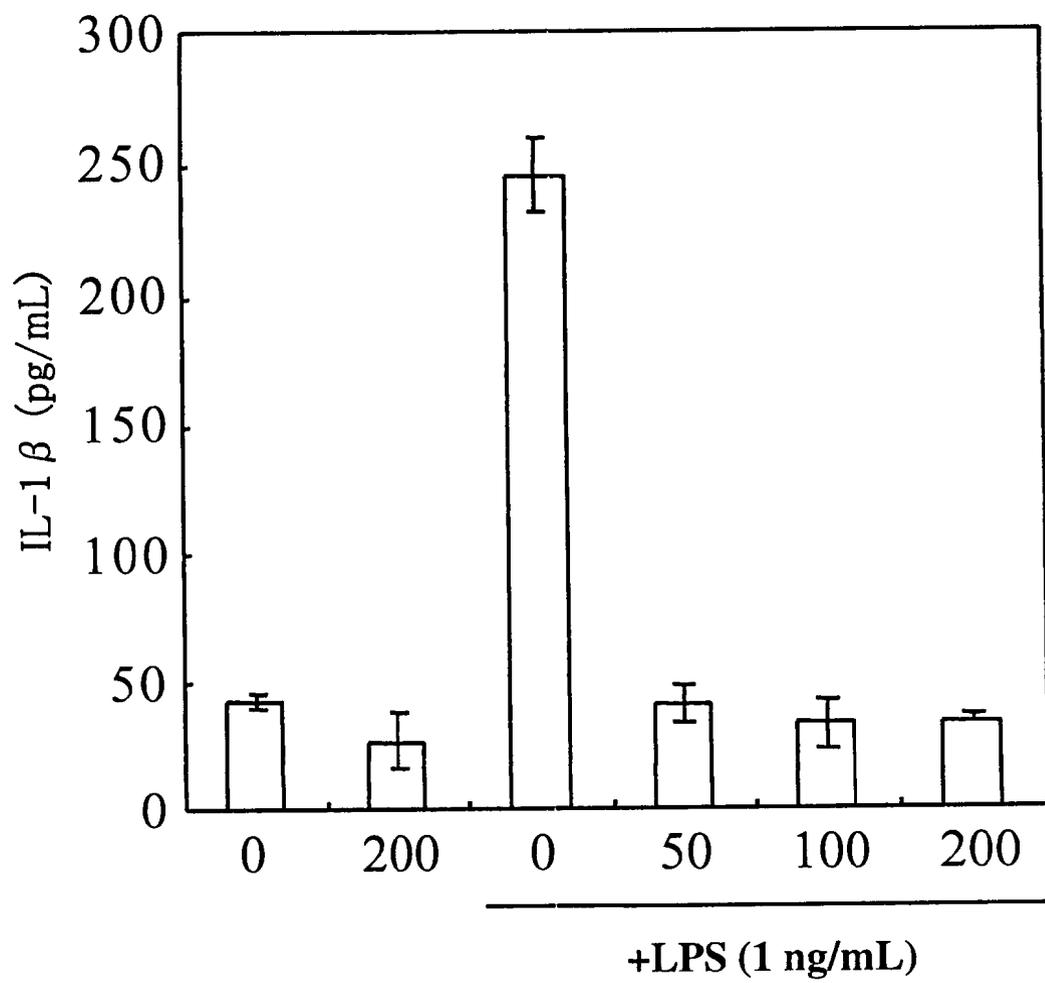


Fig.3 (c) Effects of agarobiose on LPS-induced IL-1  $\beta$

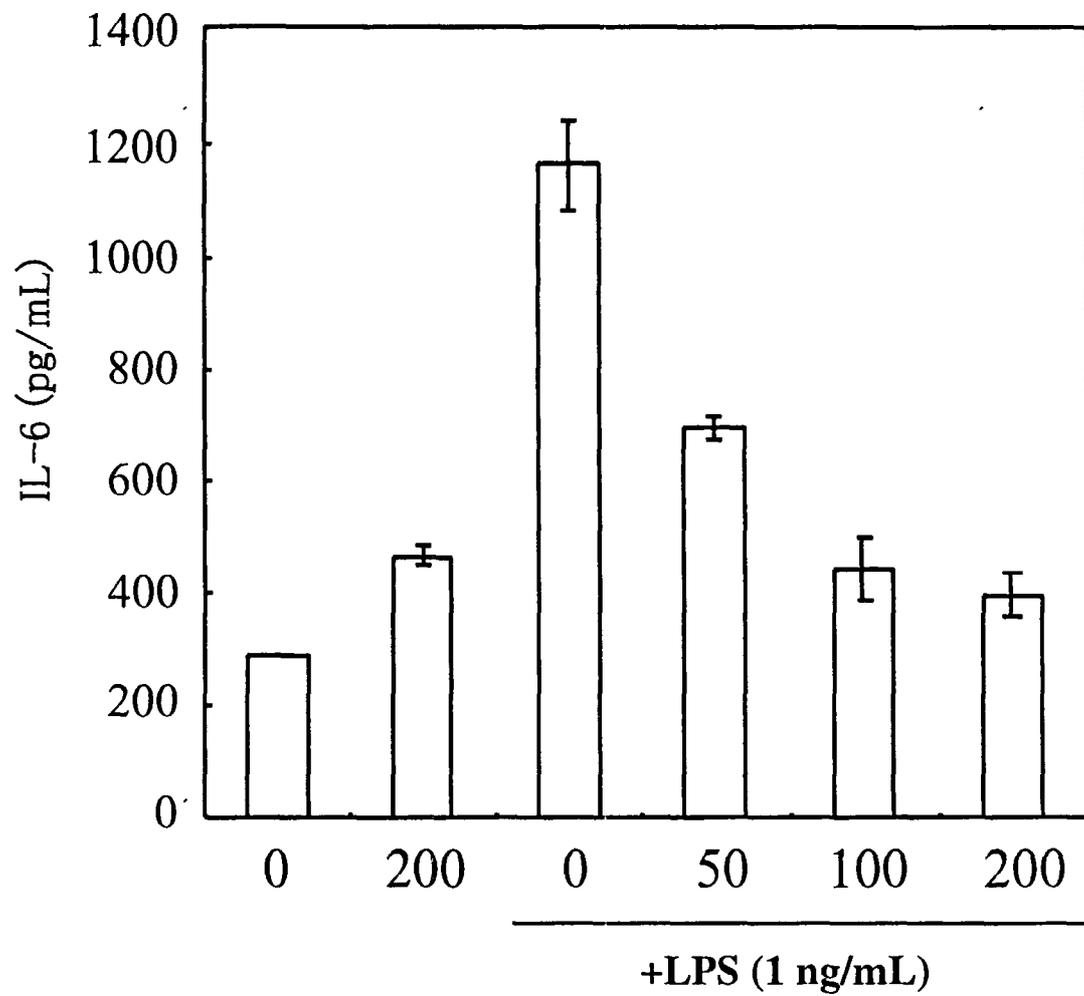
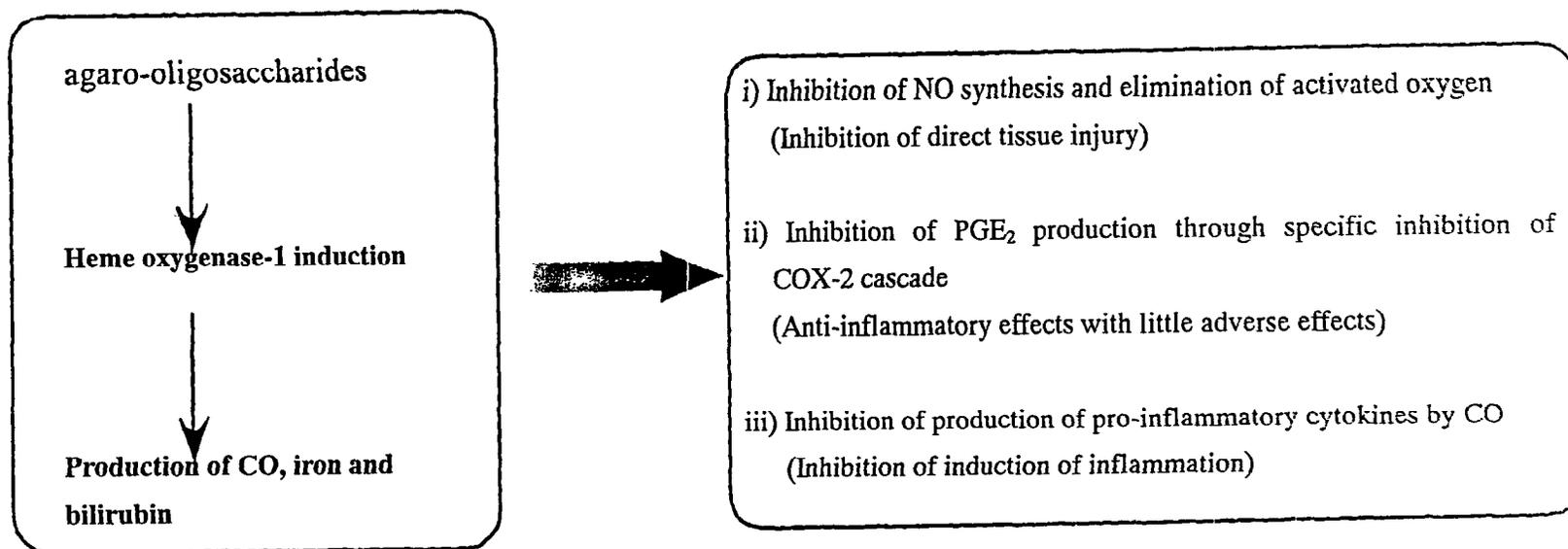


Fig.3 (d) Effects of agarobiose on LPS-induced IL-6



**Fig.4 Possible Site of Action of Agaro-oligosaccharides in the Prevention and Treatment of RA**

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## **Anti-inflammatory Effects of Agaro-Oligosaccharides**

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### **Introduction**

Kanten (agar), Iwanori, and Sushinori (*Porphyra tenera*) are representatives of red seaweed, which have been a food substance forming a key part of the everyday Japanese diet from ancient times. Over the last several years, the Biotech Research Laboratory of Takara Shuzo has conducted research on the functionality of agar, a representative of red seaweed<sup>1)</sup>. The main component of the polysaccharides contained in agar is agarose, which is a linear sugar chain consisting of galactose (3-O-linked  $\beta$ -D-galactopyranose: abbreviated to "Gal") and anhydrogalactose (4-O-linked 3,6-anhydro- $\alpha$ -L-galactopyranose: abbreviated to "Ah-Gal") connected alternatively. Since the  $\alpha$ 1-3 linkage is unstable, agarose is degraded under slightly acidic conditions to produce agaro-oligosaccharides including agarobiose (consisting of Gal and Ah-Gal), agarotetraose (consisting of two pairs of agarobiose in tandem), and agarhexaose (consisting of three pairs of agarobiose). In other words, agarose is readily degraded by gastric juice to agaro-oligosaccharides, which are further converted to DGE, an active molecule, in the intestine<sup>1)</sup>. In addition, agaro-oligosaccharides are very readily uptaken by hepatocytes because their nonreducing terminal is always Gal.

Many marvelous biological activities of agaro-oligosaccharides, which had never attracted special attention before, have been revealed in the course of our investigation. These activities include: the suppression of the overproduction of nitric monoxide (NO)<sup>2)</sup>, which is involved in the enhancement of the inflammatory response and may be considered to be one of the major subjects of research in modern medicine; enhancement of the production of carbon monoxide (CO)<sup>3)</sup>, which suppresses the inflammatory response; suppression of the production of TNF- $\alpha$ <sup>4)</sup>, which is deeply involved in the worsening of rheumatism. This article considers these activities in

more detail.

### **1. Production of nitric oxide (NO) and physiological function**

Surprisingly, nitric oxide (NO), an air pollutant, plays an important role in the living body. This NO is synthesized by NO synthetase (NOS), using L-arginine as the starting material. It has been confirmed<sup>5)</sup> by cloning that NOS has three isozymes, NOS-1, NOS-2, and NOS-3.

Of these, nNOS (neuronal NOS or NOS-1) is present in the cerebral and central nervous system, iNOS (Inducible NOS or NOS-2) in macrophages, and eNOS (endothelial NOS or NOS-3) in vascular endothelial cells. Especially, eNOS and nNOS are called calcium-dependent constitutive NOS, which constitutively produces NO free radicals for signal transmission in vascular and central nervous system cells and totally regulates these systems.

On the other hand, iNOS is produced by macrophages, and its expression is induced by cytokines and lipopolysaccharide (LPS). The produced NO protects the body by destroying foreign matters oxidatively and by regulating the cell growth in the blood and immune systems. In any case, iNOS is rarely expressed under a steady state, and is induced only when stimulated by cytokines and LPS<sup>6)</sup>.

However, overproduction of this inducible NOS (iNOS) occasionally destroys the vital function. For example, the damage to the dopaminergic neurons observed in Parkinson's disease is considered to occur when the iNOS level in brain glial cells increased for some reason, or when excessive NO is produced by iNOS in macrophages infiltrated into tissues in response to the cell damage<sup>7)</sup>. In other words, it is considered that excessive NO reacts with superoxide extracellularly, forms peroxynitrite, causes damages to surrounding nerve cells, and finally destroys the nervous function. In addition, mad cow disease, which is feared around the world, is considered to be associated with NO and oxygen radicals<sup>8)</sup>.

It is known that the risk of developing atherosclerosis, which is a major cause of heart diseases, increases in inverse proportion to the blood concentration of high-density lipoprotein (HDL) cholesterol. However, the precise mechanism was not known until recently. Yuhanna et al. have discovered that HDL stimulates and activates the eNOS of vascular endothelial cells<sup>9)</sup>. On the other hand, no activation of eNOS occurred with low-density lipoprotein (LDL). It was clarified that both eNOS and scavenger

receptor-BI (SR-BI) are co-localized in the specific sites of vascular endothelial cells and that eNOS is activated by the binding of HDL apolipoprotein (ApoA-I) to SR-BI.

## **2. Production of carbon monoxide (CO) and physiological function**

### **(1) Production of anti-oxidant**

In the living body, heme receives catabolism by heme oxygenase (HO) to yield equimolar quantities of carbon monoxide (CO), bilirubin, and iron. As in the case of NOS, HO has three isoforms. Of these isoforms, HO-2 and HO-3 are the constitutive type, and HO-1 is the inducible type, possessing a potent anti-inflammatory effect and also protecting the body from oxidative stress<sup>10</sup>. HO-1 is induced by heavy metals, various cytokines, hormones, endotoxins, heat shock, etc. In other words, HO-1 is strongly inducible by hydrogen peroxide, ultra violet, hyperoxemia, and other substances inducing oxidative stress. HO-1 is considered to maintain homeostasis by protecting cells and tissues from the damage induced by oxidants<sup>11</sup>. In rats in which the genes of HO-1 were introduced into the lungs using an adenovirus-derived vector, no damage was produced in the lungs even when the O<sub>2</sub> level was higher than 99%<sup>12</sup>. Therefore, the mechanism to protect cells from oxidative stress appears to be associated with the action of anti-oxidants such as bilirubin, ferritin, and CO, which is the major product from the catabolism of heme.

### **(2) Suppression of TNF- $\alpha$ production**

It is not widely known that sepsis is a horrible disease for inpatients and is the principle cause of death. Death is caused not directly by the causative pathogens of the infection, but by the abrupt decrease in blood pressure due to the vasodilation caused by the induced NO, or by uncontrollable overproduction of TNF- $\alpha$ , IL-1 $\beta$ , macrophage inflammatory protein MIP-1 $\beta$ , and other proinflammatory cytokines and chemokines. Regarding the latter cause, such overproduction is known to cause increased white blood cells, leakage of peripheral blood vessels, and the destruction of tissues.

LPS, which is the main trigger of sepsis, is one of the cell wall components of Gram-negative bacteria. In mice (or macrophages) treated with LPS, severe symptoms such as those obtained in sepsis appear. However, anti-inflammatory cytokines such as IL-10 and IL-4 are produced some time later and exert their actions to alleviate the inflammation by inhibiting the synthesis of proinflammatory cytokines and chemokines.

When macrophage RAW264.7 cells are treated with LPS, the TNF- $\alpha$  production starts as expected. Choi et al. reported that TNF- $\alpha$  production in response to LPS was 5-fold higher in a macrophage cell line that does not express HO-1 than in another cell line that genetically expresses a large amount of recombinant HO-1<sup>3)</sup>. This demonstrates that HO-1 significantly suppresses the TNF- $\alpha$  production.

Furthermore, it was found that the production of TNF- $\alpha$  decreases with the increasing concentration of CO when a macrophage cell line was treated with a trace amount of LPS in the presence of CO gas. In addition, in the presence of CO, the production of IL-1 $\beta$  and MIP-1 $\beta$  were also decreased. These results were also demonstrated in vivo experiments conducted in mice.

Next, Choi et al. investigated the LPS-induced TNF- $\alpha$  production in the presence/absence of CO in cells in which NO production was inhibited by the treatment with L-NAME, a NOS-specific inhibitor. They found that TNF- $\alpha$  production was not affected by L-NAME. In addition, CO was found to have no influence on LPS-induced NO production. These results have demonstrated that the suppression of TNF- $\alpha$  production is not the result of the effect of CO on the pathway involved in NO production.

### **3. Functionality of agaro-oligosaccharides**

#### **(1) Suppression of the inducible nitric monoxide synthetase (iNOS) expression**

Our world first discovery is that, in mouse peritoneal macrophages treated with lipopolysaccharide (LPS) and interferon gamma (IFN- $\gamma$ ), the NO production increases due to the enhancement of iNOS expression, but in the presence of agarobiose the NO production decreases in inverse proportion to the amount of agarobiose (Fig. 1)<sup>13)</sup>. Western blotting analysis using an anti-iNOS monoclonal antibody demonstrated that this decrease in NO production was caused by the decreased iNOS protein in response to agarobiose (Fig. 2). Next, we investigated the induced expression of iNOS mRNA following the pretreatment with agarobiose. The result showed stronger inhibition on the iNOS mRNA expression in proportion to the duration of agarobiose pretreatment (Fig. 3), demonstrating that agarobiose inhibits the iNOS mRNA expression.

Neoagarobiose, in which Gal and Ah-Gal are joined in the reverse manner to those of agarobiose, did not show any of those effects mentioned above.

## (2) Suppression of prostaglandin E<sub>2</sub> production and TPA-induced edema

It has been confirmed that prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is responsible for the following symptoms observed in joint inflammation: redness, swelling, hotness, and pain.

It was found that LPS-induced PGE<sub>2</sub> production during 18 hours post-induction was about 20% of the control level in the mouse macrophage cell line, RAW264.7, when the cells were pretreated for 5 hours with agarohexaose. According to the reigning theory, PGE<sub>2</sub> is normally produced from arachidonic acid by cyclooxygenase. However, when arachidonic acid was added, PGE<sub>2</sub> production was generally increased even in the presence of agaro-oligosaccharides, indicating that agaro-oligosaccharides do not inhibit cyclooxygenase. Therefore, it appears that agaro-oligosaccharides inhibit PGE<sub>2</sub> production through an unknown novel passway<sup>14)</sup>.

TPA, one of the phorbol esters, possesses an important effect that leads to tumor promotion by enhancing the cellular phospholipid metabolism. Therefore, suppressing the effect of TPA is regarded to be very important as an anti-tumor promoter. This tumor promoter TPA induces edema when applied to the ear in mice. It has been demonstrated that the oral treatment with a 10% agaro-oligosaccharide or its external application to the ear for 14 days prior to the application of TPA suppressed the induction of edema<sup>15)</sup>. The effect of agaro-oligosaccharides was investigated in mice on papilloma induced by the administration of 100 µg DMBA, a carcinogenic substance, followed, from 1 week later, by treatment with 1 µg TPA applied over the dorsal skin twice weekly for a period of 20 weeks. Agaro-oligosaccharide solutions were given to those mice instead of drinking water, starting 1 week before the administration of DMBA. In those receiving the 3% agaro-oligosaccharide solution, the number of mice that developed papilloma was about 10% of the corresponding number obtained in the control group (receiving water) (Fig. 4).

## (3) Expression of inducible carbon monoxide synthetase (HO-1)

Here, carbon monoxide synthetase means heme oxygenase. As mentioned earlier, heme undergoes catabolism by heme oxygenase (HO) and yields equimolar quantities of carbon monoxide (CO), bilirubin, and iron. The macrophage RAW264.7 cells did not express HO-1 after being cultured for 12 hours in a culture medium alone. Treatment of the cells with LPS alone or with LPS plus IFN-γ increased HO-1 expression slightly,

whereas the addition of agarobiose to the culture increased HO-1 expression in a concentration-dependent manner (Fig. 5)<sup>16</sup>. This is considered to be the result of the synergistic effect of agarobiose and the oxidative stress caused by LPS-induced NO. On the other hand, in addition to HO-1, other stress response proteins HSP-70 and GRP-78 were also investigated and found to be unaffected. This indicates that this effect is specific to NO and agarobiose.

#### (4) Suppression of TNF- $\alpha$ production

A study was carried out in mice that were allowed to have free access to an aqueous solution of heated agar as drinking water for 19 days. After 19 days, a high dose of LPS (300  $\mu\text{g}/\text{mouse}$ ) was given intraperitoneally to induce endotoxin shock. The mortality rate in the group treated with 1% agar solution was 100% (8/8 mice), whereas the rate in the group treated with 10% agar solution was 25% (2/8 mice). This clearly indicates the protective effect of agar against endotoxin shock. In the same manner, a low dose of LPS (20  $\mu\text{g}/\text{mouse}$ ) was given intraperitoneally to mice that had been treated with the agar solutions for 19 days. One hour after administration, the serum level of TNF- $\alpha$  was determined. In those treated with the 10% solution, the TNF- $\alpha$ -level was found to be lower by about 30%.

In another experiment, mouse macrophages were cultured in the presence of agarobiose for 6 hours, and then LPS was added to the culture to make the final concentration of 1  $\mu\text{g}/\text{mL}$ . Twenty-four hours later, the amount of TNF- $\alpha$  produced was determined (Fig. 6)<sup>17</sup>. In the presence of 200  $\mu\text{M}$  agarobiose, TNF- $\alpha$  production decreased by about 60%. These experiments were also carried out in monocytes derived from human peripheral blood mononuclear cells. In the human monocytes, the decrease in TNF- $\alpha$  production was observed with a lower concentration of agarobiose than in mice (Fig. 7).

#### **4. Use as a supplement for preventing arthroseitis**

Health food supplements are available for various diseases. Chondroitin sulfate and glucosamine are available as supplements for arthroseitis in the U.S. and held a 120-billion dollar market last year. The sole rationale for the indication that these compounds are effective against arthroseitis is that these two compounds are the constituents of human joint cartilage. In the 1980s, it was reported that these

compounds relieve the pain associated with arthroseitis, and the National Institute of Health (NIH) in the U.S. started a 4-year research project with a grant of about 7 million dollars.

As mentioned above, the oral administration of agaro-oligosaccharide derived from agar suppresses the expression of proinflammatory cytokines such as TNF- $\alpha$ , which is considered to be a cause of rheumatism, enhances the expression of heme oxygenase-1, and produces various molecules possessing anti-oxidative effects, such as carbon monoxide. In fact, it is said that a solidified agar solution is used habitually as a folk medicine effective against rheumatism in Peru and the surrounding countries in South America. In Tanabe Seiyaku Co., Ltd., a therapeutic drug for rheumatism (brand name: Remicade) is being developed from an anti-TNF- $\alpha$  antibody (in Phase II/III).

Taken together, it is highly likely to be demonstrated that agaro-oligosaccharides are effective against a certain type of rheumatism.

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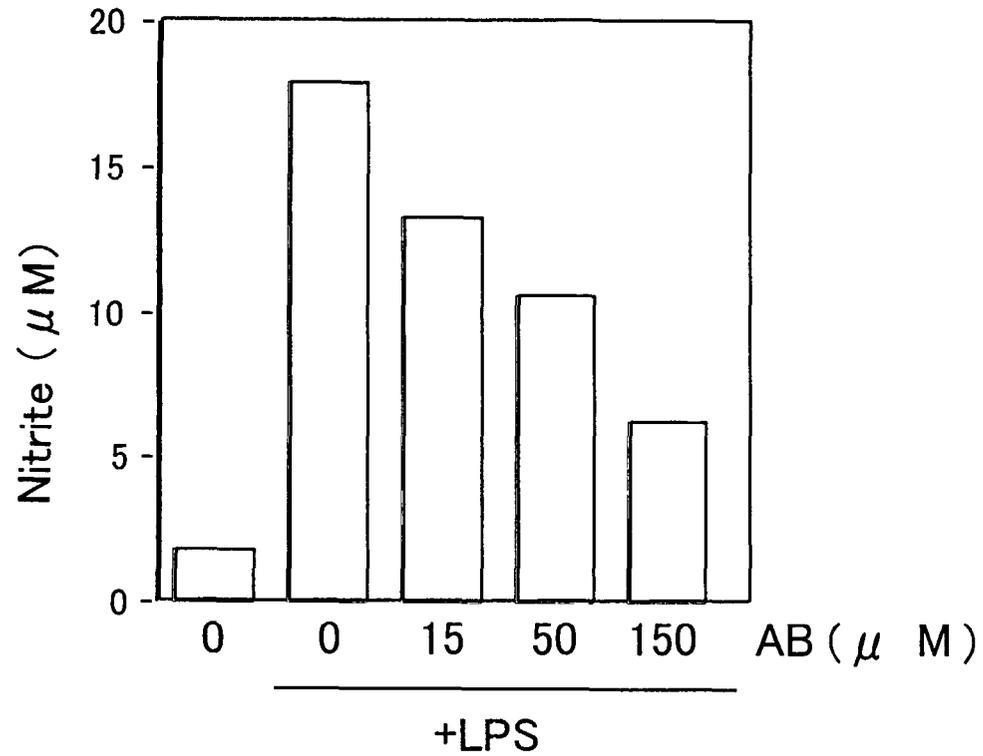


Fig.1 Effect of Agarobiose on NO production by mouse peritoneal macrophages

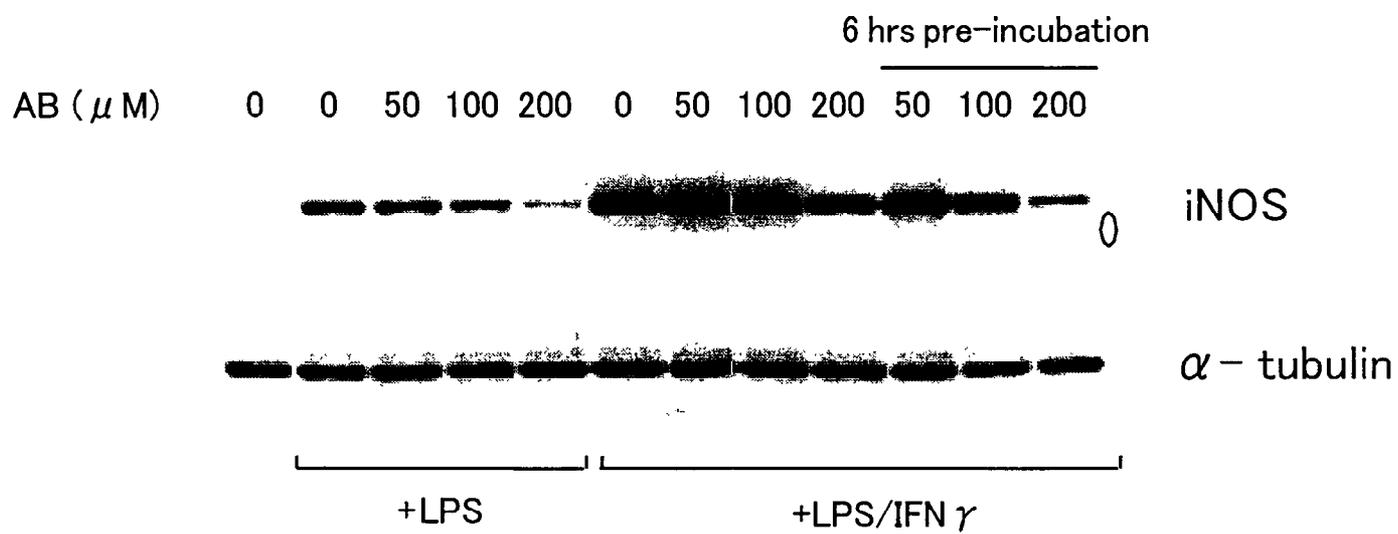


Fig.2 Suppression of iNOS protein expression by Agarobiose

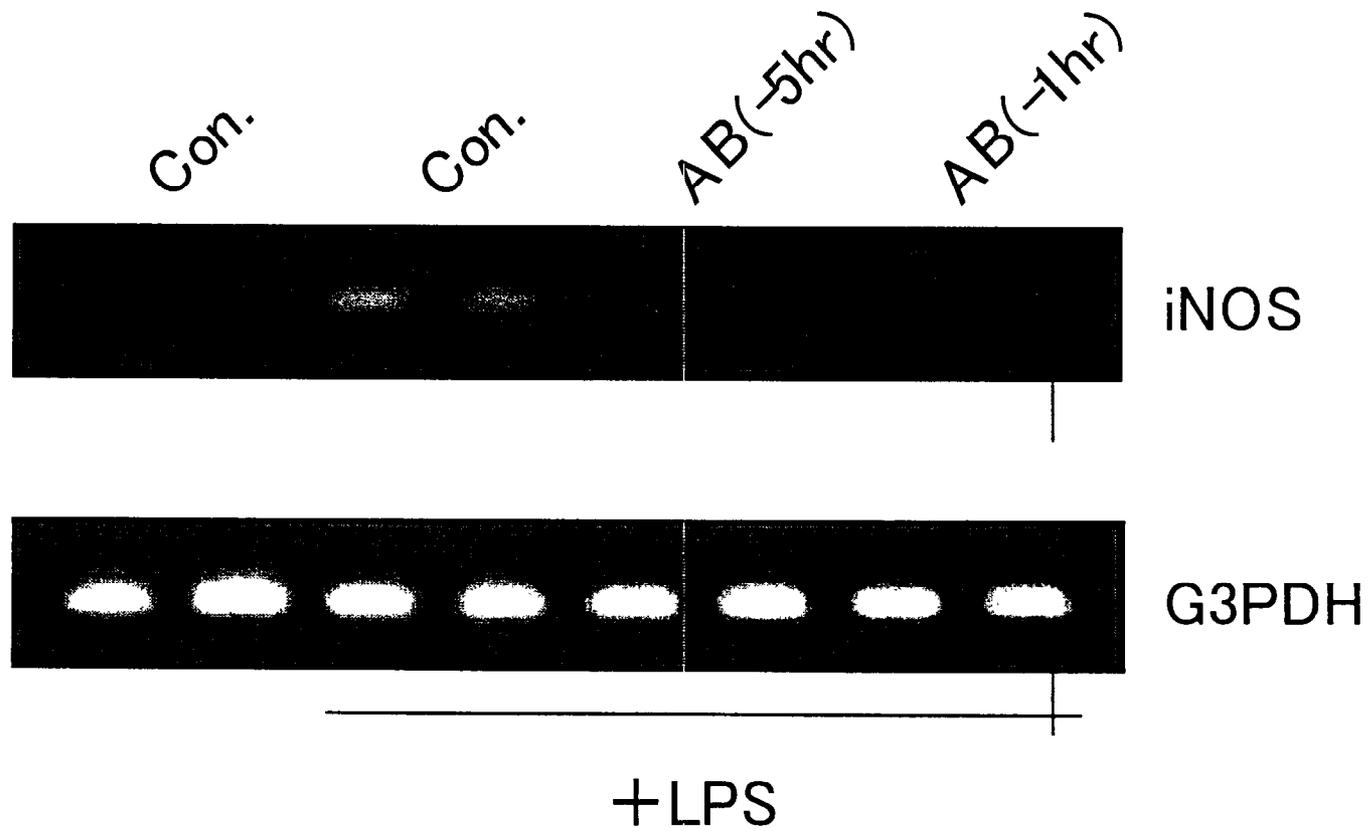
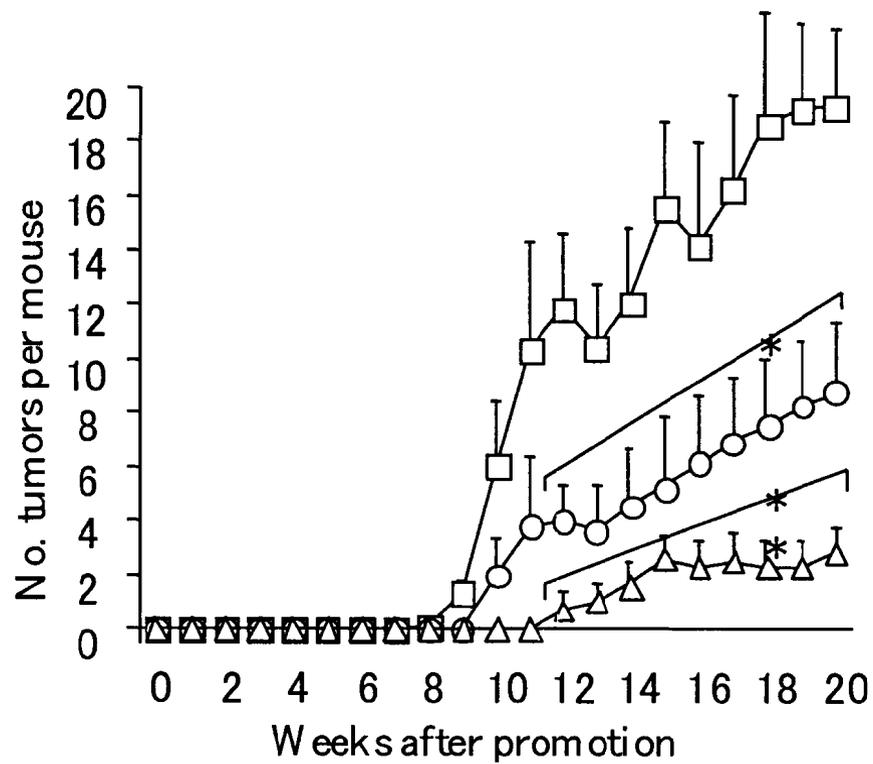


Fig.3 Suppression of iNOS mRNA expression by Agarobiose



□ Control      ○ 1% Agar-oligosaccharides      △ 3% Agar-oligosaccharides

Data show mean  $\pm$  SEM of 10 animals.    \*:  $p < 0.05$ , \*\*:  $p < 0.01$  vs control

**Fig.4 Preventive effect of Agar-oligosaccharide on cancer development**

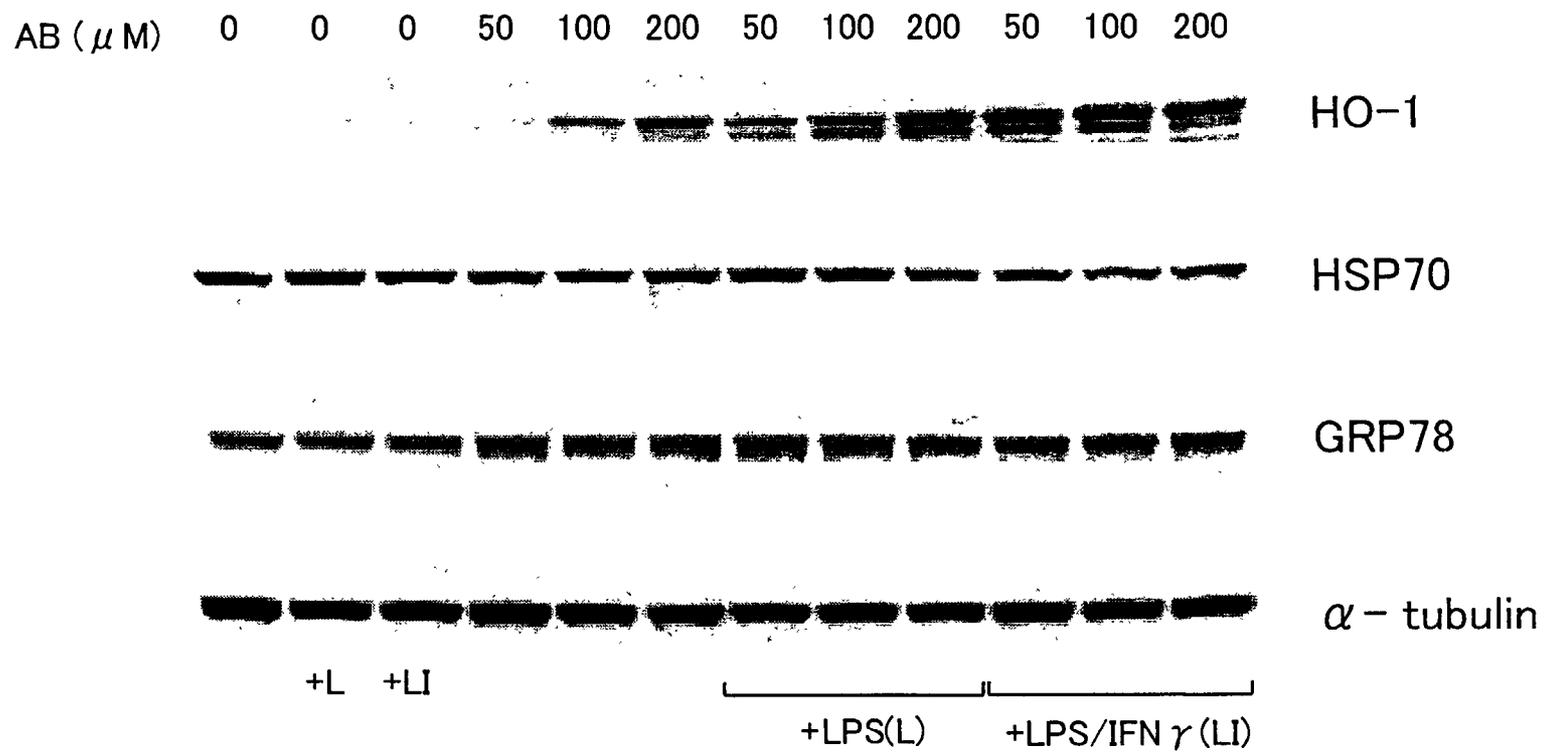


Fig.5 Induction of HO-1 expression by Agarobiose

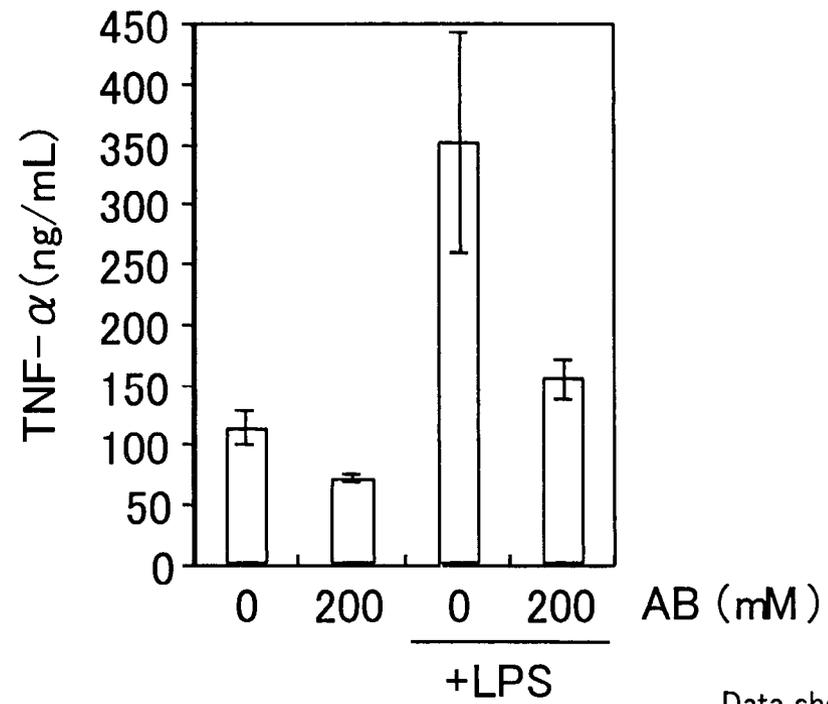


Fig.6 Effect of Agarbiose on TNF- $\alpha$  production by mouse peritoneal macrophage

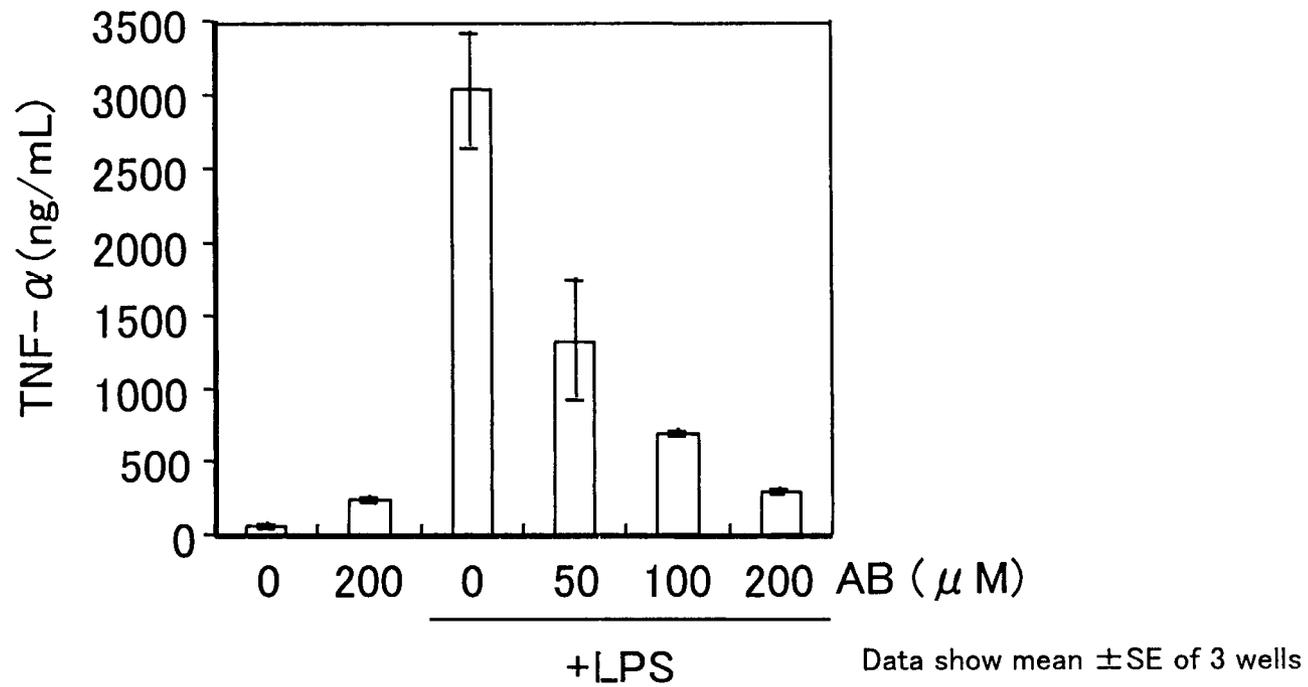


Fig.7 Effect of Agarobiose on TNF- $\alpha$  production by the monocytes derived from human peripheral blood mononuclear cells

## Publication of Study on Agaro-oligosaccharide (1998–2000)

Biotech. Res. Lab., Takara Shuzo Co.,Ltd.

	Academic society	Period	Place	No	Subject	Speakers
1	The 20th Japanese Carbohydrate Symposium	1998.7.15–17	Sapporo	B1-05	Agaro-oligosaccharides induce apoptosis in HL-60 cells and suppress nitric oxide production in macrophage	Tatsuji Enoki, Shinji Okuda, Hiroaki Sagawa, Katsushige Ikai and Ikunoshin Kato
2	The 57th Annual Meeting of the Japanese Cancer Association	1998.9.30–10.2	Yokohama	2232	Effect of Agaro-oligosaccharides from red algae on proliferation and nitric oxide production in mammalian cell lines	Tatsuji Enoki, Hiroaki Sagawa and Ikunoshin Kato
3	The 71th Meeting of the Japanese Biochemical Society	1998.10.14–17	Nagoya	3N-W29-06	Agaro-oligosaccharides induce apoptosis in HL-60 cells and suppress nitric oxide production in macrophage	Tatsuji Enoki, Shinji Okuda, Hiroaki Sagawa, Katsushige Ikai and Ikunoshin Kato
4	The 6th Japanese Society for Cancer Prevention	1999.7.16–17	Tokyo	P-37	Basic studies on Agaro-oligosaccharides as cancer-preventive food product	Tatsuji Enoki, Shinji Okuda, Takanari Tominaga, Hiroaki Sagawa and Ikunoshin Kato
5	The 58th Annual Meeting of the Japanese Cancer Association	1999.9.29–10.1	Hiroshima	246	Inhibitory effects of Agaro-oligosaccharides on NO and PGE <sub>2</sub> generation in activated-macrophages	Tatsuji Enoki, Takanari Tominaga, Hiroaki Sagawa and Ikunoshin Kato
6	The 58th Annual Meeting of the Japanese Cancer Association	1999.9.29–10.1	Hiroshima	247	Effect of Agaro-oligosaccharides analogs on proliferation and NO synthesis in mammalian cells lines	Eiji Kobayashi, Tatsuji Enoki, Hiroaki Sagawa and Ikunoshin Kato
7	The 58th Annual Meeting of the Japanese Cancer Association	1999.9.29–10.1	Hiroshima	248	Anti-inflammatory action of Agaro-oligosaccharides on TPA-induced inflammation in mice	Takanari Tominaga, Eiji Nishiyama, Tatsuji Enoki, Hiroaki Sagawa, Shigetoshi Mizutani and Ikunoshin Kato
8	The 58th Annual Meeting of the Japanese Cancer Association	1999.9.29–10.1	Hiroshima	2258	Anti-tumor activity of Agaro-oligosaccharides from agar against human colon cancer xenografts	YU FU-gong, Takeshi Sakai and Ikunoshin Kato
9	The 72th Meeting of the Japanese Biochemical Society	1999.10.6–9	Yokohama	2P-001	Preventive and therapeutic effect of Agaro-oligosaccharides in acute and chronic inflammation models	Eiji Nishiyama, Suzu Deguchi, Kinya Fujii, Hiroaki Sagawa, Shigetoshi Mizutani and Ikunoshin Kato
10	The 72th Meeting of the Japanese Biochemical Society	1999.10.6–9	Yokohama	2P-115	Novel $\alpha$ -agarose derived from seaweed and microorganisms	Jun Tomono, Keiko Nomura, Hiroaki Sagawa, Takeshi Sakai and Ikunoshin Kato
11	The Annual Meeting of the Japan Society for Bioscience, Biotechnology and Agrochemistry in 2000	2000.3.31–4.2	Tokyo	3D040 $\beta$	Novel $\alpha$ -agarose produced by marine microorganisms	Jun Tomono, Keiko Nomura, Takeshi Sakai, Hiroaki Sagawa and Ikunoshin Kato

## Publication of Study on Agaro-oligosaccharide (1998-2000)

Biotech. Res. Lab., Takara Shuzo Co.,Ltd.

	Academic society	Period	Place	No	Subject	Speakers
12	The Annual Meeting of the Japan Society for Bioscience, Biotechnology and Agrochemistry in 2000	2000.3.31-4.2	Tokyo	2E043 $\beta$	Correlation between induction of HO-1 and suppression of nitric oxide production by Agaro-oligosaccharides	Tatsuji Enoki, Hiroaki Sagawa and Ikunoshin Kato
13	The 59th Annual Meeting of the Japanese Cancer Association	2000.10.4-6	Yokohama	1927	Correlation between induction of HO-1 and various physiological activity by Agaro-oligosaccharides, and analysis of gene expression with DNA-chip technology	Tatsuji Enoki, Hiroaki Sagawa and Ikunoshin Kato
14	Japanese Association for Dietary Fiber Reseach, 5th Reseach Meeting	2000.11.17-18	Tokyo	4	Polysaccharides from Seaweeds : Agarose and Fucoidans as Functional Foods	Ikunoshin Kato