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Original Article

Safety assessment of a soluble dietary fiber, isomaltodextrin, enzymatically produced from starch

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ABSTRACT — A series of safety studies were conducted using isomaltodextrin (IMD), a new dietary fiber that is produced from starch using enzymes. IMD consists of only alpha linkaged glucose molecules, has an average molecular weight of approximately 5,000, is freely soluble in water, and contains greater than 80% fiber (AOAC 2001.03). No genotoxicity was observed when IMD was assayed in standardized bacterial reverse mutation, micronucleus, and chromosome aberration tests. The LD_{50} of IMD was found to be more than 2,000 mg/kg in an acute toxicity study in rats, and the no observed adverse effect level (NOAEL) was determined to be 1,000 mg/kg/day in a 90-day subacute gavage toxicity study in rats. No animals died, and no abnormal findings due to consumption of IMD were observed in either of these studies. Both NOAEL values were the highest doses tested. The NOAEL for loose stools was examined in humans in two separate studies. Based on these results, the NOAEL for IMD-related loose stools was considered to be 0.8 g/kg-BW. In a 4-week high-dose ingestion study in humans and a 12-week low-dose ingestion study in humans, laboratory values were found to be within the normal range of variation. The results of the current safety assessment studies suggest that isomaltodextrin is safe for human consumption.

Key words: Isomaltodextrin, Genotoxicity, Animal feeding study, Human consumption study

INTRODUCTION

Isomaltodextrin (IMD) is a highly branched α-glucan produced enzymatically from starch using commercially available thermostable α-amylase and α-amylase, and α-glucosyltransferase and α-amylase derived from *Paenibacillus alginolyticus* PP710 (Tsusaki *et al.*, 2012). IMD is a white powder with no odor, it is freely soluble in water and dissolves into a clear solution, and therefore is unlikely to alter the smell, color or taste of a product in which it is used. The dietary fiber content of IMD is more than 80% (Tsusaki *et al.*, 2009). Therefore, IMD is expected to be applicable for widespread use in foods as a new soluble dietary fiber.

IMD contains only glucose units and alpha bonds that consist of approximately the following amounts of glucosidic linkages: 17% α-1 (nonreducing end groups), 3% α-1,3, 19% α-1,4, 49% α-1,6, 7% α-1,3,6, and 5% α-1,4,6 (Tsusaki *et al.*, 2012). The average molecular weight is approximately 5,000 (Fig. 1). There have been no reports of a glucan containing all of these linkages; however, resistant maltodextrin, resistant dextrin and polydextrose, three well known soluble dietary fibers, are also polymers of glucose and have α-1,6, α-1,4 and α-1,3 glucosidic linkages. There have been numerous safety studies conducted on these types of glucose polymers that have dietary fiber function. The safety of resistant maltodextrin has been confirmed in acute toxicity, mutagenicity (Wakabayashi *et al.*, 1992), human gastrointestinal tolerance (Kishimoto *et al.*, 2013), and human ingestion studies (Kajimoto *et al.*, 2001; Inafuku *et al.*, 2004). The safety of resistant dextrin has also been reported in acute toxicity, 90-day feeding toxicity, mutagenicity (Wils *et al.*, 2008), and human consumption studies (van den Heuvel *et al.*, 2004; Pasman *et al.*, 2006). The safety of polydextrose has been shown in a number of similar animal and human consumption studies (Burdock and Flamm, 1999; Takano *et al.*, 2015). It is believed that because of the structural similarities between IMD, and resistant maltodextrin, resistant dextrin and polydextrose that these substances are substan-

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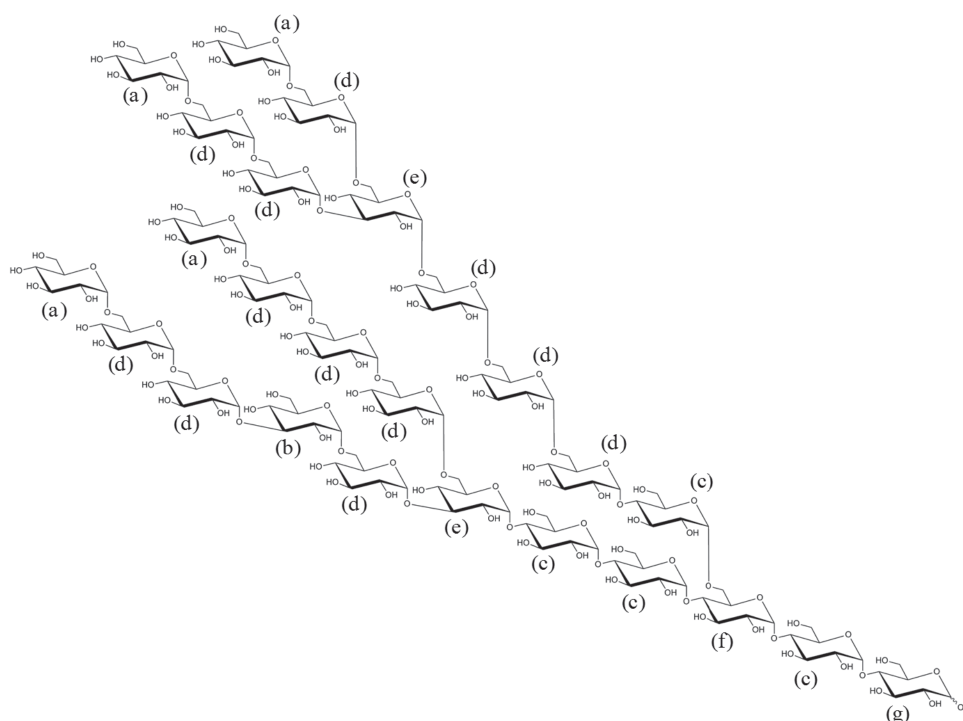

Fig. 1. The putative structure of IMD. (a) Nonreducing-end α-Glcp; (b) 1,3-linked α-Glcp; (c) 1,4-linked α-Glcp; (d) 1,6-linked α-Glcp; (e) 1,3,6-linked α-Glcp; (f) 1,4,6-linked α-Glcp; and (g) reducing-end Glcp.

tially equivalent and therefore would have a similar safety profile. However, to insure the specific safety of IMD, a number of *in vitro* and *in vivo* safety studies were conducted. These included 3 types of genetoxicity studies, acute and subchronic feeding studies in rats, and four human studies (two tolerance studies, a 4-week high-dose ingestion study, and a 12-week low-dose feeding study).

MATERIALS AND METHODS

Test substance

IMD (Fibryxa; Hayashibara Co., Ltd., Okayama, Japan), the test substance, had a dietary fiber content of between 80.8% and 82.9% as determined by an enzymatic/HPLC method (AOAC 2001.03).

Animals

Rats (Crl:CD [SD], SPF) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Animals were individually housed in wire-mesh cages in a licensed animal room. The room was controlled at a temperature range of 20 to 26ºC, and a relative humidity of 35 to 70%, with a ventilation frequency of not less than 12 volumes exchanged per hour, and a 12-hr light cycle (lights on from 7:00 am to 7:00 pm). Animals were allowed access to pellet diet CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan) and city water *ad libitum*.

Ethics

Animal experiments were performed in compliance with Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notice No.88, Japanese Ministry of the Environment, April 28, 2006).

The human studies were conducted on the basis of the principles of the Declaration of Helsinki, and were approved by the Ethics Committee of Hayashibara Co., Ltd. The 4-week and 12-week ingestion studies in humans were also approved by the Ethics Committee of the Miura Clinic (Osaka, Japan).

Bacterial reverse mutation test

A bacterial reverse mutation test was performed according to the OECD Guideline for the Testing of Chemicals (OECD, 1997a). *Salmonella typhimurium* strains (TA98, TA100, TA1535 and TA1537) and a *Escherichia coli* strain (WP2*uvrA* [pKM101]) were purchased from Molecular Toxicology, Inc. (Boone, NC, USA). Rodent liver homogenate (S9) and Cofactor-A were purchased

from Oriental Yeast Co., Ltd. S9 mix was prepared by mixing S9 and Cofactor-A at a ratio of 1:9 immediately before use. All experiments were performed in the presence and absence of the S9 mix. This study was conducted according to the pre-incubation method. All treatments were divided into two groups, and each group was examined in the absence and presence of S9 mix. This study was performed twice. The bacterial strains were treated with IMD at concentrations of 313, 625, 1,250, 2,500, and 5,000 µg/plate. Also, positive and negative control substances were tested concurrently. Water served as a negative control for all experiments. The following compounds were employed as positive controls: sodium azide (SA), 2-nitrofluorene (2-NF), 2-aminoanthracene (2-AA), 9-aminoacridine (9-AA), and 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF2). The above compounds, except for AF2 (positive control), were purchased from Sigma-Aldrich (St. Louis, MO, USA). AF2 was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The positive controls were set as shown in Table 1. A sample was considered positive if it met the following criteria, "The number of revertant colonies in any strains at one or more doses is increased at least twice the negative control value. It should be increased in a dose-dependent manner and with reproducibility." A sample was considered negative if it didn't meet the preceding criteria.

Micronucleus test

A mammalian erythrocyte micronucleus test was performed according to the OECD Guideline for the Testing of Chemicals (OECD, 1997b). Thirty rats aged 8 weeks were used for the test. Three dose levels, including the highest dose of 2,000 mg/kg, as specified in the guideline, and lower doses of 1,000 and 500 mg/kg, were used. Water and mitomycin C (MMC, Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) at concentrations of 2 mg/kg were used as negative and positive controls, respectively. The dosing volume was 1 mL per 100 g-body weight (BW). IMD and the negative control were orally administered to 6 male rats per dose group once a day for 2 consecutive days. The positive control was injected once via the tail vein using a disposable syringe with a 25-gauge needle on the day before preparation of bone marrow samples. At 24 hr after the final administration, body weights of the animals were measured before preparation of bone marrow samples. The frequency of micronucleated immature erythrocytes (MNIE) and the ratio of immature erythrocytes (IE) to the total number of analyzed erythrocytes were calculated for each group. In the IMD-treated groups and negative control group, clinical signs of the animals were observed at 1, 24 and 25 hr after the first dosing, and just before preparation of bone marrow samples (48 hr after the first dosing). In the positive control group, clinical signs were observed at 1 hr after dosing and just before preparation of bone marrow samples (24 hr after dosing). To evaluate the results it was first confirmed whether the frequency data of MNIE in each test substance group were within the acceptable ranges (mean ± 3 S.D.) calculated from historical data of other negative control groups. A test result was considered positive if a statistically significant increase in the frequency of MNIE was observed in the test substance groups as compared with that in the negative control group. The final judgment was made taking into consideration the biological relevance under the test conditions.

Chromosome aberration test

A mammalian chromosome aberration test was performed according to the OECD Guideline for the Testing of Chemicals (OECD, 1997c). A Chinese hamster lung fibroblast cell line (CHL/IU) was purchased from the National Institute of Hygienic Sciences (Tokyo, Japan).

Table 1. Bacterial strains, and positive controls and doses used in the bacterial reverse mutation test.

S9 mix	Strains	Positive controls*	Doses (µg/plate)
-	TA98	2-NF	5.0
	TA100	SA	1.5
	TA1535	SA	1.5
	TA1537	9-AA	80.0
	WP2*uvrA*(pKM101)	AF2	0.005
+	TA98	2-AA	1.0
	TA100	2-AA	2.0
	TA1535	2-AA	3.0
	TA1537	2-AA	3.0
	WP2*uvrA*(pKM101)	2-AA	2.0

*2-NF, 2-nitrofluorene; SA, sodium azide; 9-AA, 9-aminoacridine; AF2, 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide, 2-AA; 2-aminoanthracene.

The maximum concentration of IMD was 5,000 µg/mL as prescribed in the test guideline, and concentrations of 2,500, 1,250 and 625 µg/mL were also used for the test. S9 and Cofactor-C were purchased from the Oriental Yeast Co., Ltd. (Tokyo, Japan), and S9 mix was prepared immediately before use. The CHL/IU cells were treated with IMD or control substances under the following conditions: 1) short-term treatment (6 hr) in the absence of S9 mix followed by an 18-hr recovery period (-S9 assay); 2) short-term treatment (6 hr) in the presence of S9 mix followed by an 18-hr recovery period (+S9 assay); and 3) continuous treatment for 24 hr in the absence of S9 mix (24-hr assay). Water for injection was used as the negative control, which was the solvent used to prepare the test substance solutions. Mitomycin C (MMC, Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) was used at concentrations of 0.1 and 0.05 µg/mL as a positive control for the -S9 and 24-hr assays, respectively. Cyclophosphamide (CP, Shionogi & Co., Ltd., Osaka, Japan) was used as a positive control at 12.5 µg/mL for the +S9 assay. Microscopic examinations were conducted with concentrations of IMD of 1,250, 2,500 and 5,000 µg/mL for the -S9, +S9 and 24-hr assays. The final evaluation was made on the total incidence of aberrant cells minus the number of cells with only chromosomal structural aberrant gaps. The test result was considered positive if the incidence of aberrant cells in the treated group was significantly higher (one-sided significance level: $p < 0.025$) than in the negative control group, and concentration dependency was significant (one-sided significance level: $p < 0.025$), or reproducibility could be confirmed. The final judgment was made taking into consideration the biological relevance under the test conditions.

Acute oral toxicity study in rats

An acute oral toxicity study was conducted according to the OECD Guidelines for the Testing of Chemicals (OECD, 2001). Five rats aged 6 weeks were used for the study. A single dose of 2,000 mg/kg of IMD as an aqueous solution of 200 mg/mL was administered to the rats by oral gavage following an overnight fast. The dose volume was 1.0 mL per 100 g-BW. The animals were fasted for a further 3 hr after dosing, and then allowed feed and water *ad libitum*. The general condition of the animals was observed once within 30 min and once at 1, 2, 3, and 4 hr after dosing (Day 0). For 14 days from the 1st day after dosing (from Day 1 to Day 14), the general condition of the test animals was observed once daily. The viability, as well as signs of toxicity, were assessed, and any observed effects were recorded. Body weights were recorded on Days 0 (before dosing), 7 and 14. All animals were sacrificed on Day 14 and the organs/tissues were examined.

90-day repeated-oral dose toxicity study in rats

A 90-day repeated-oral dose toxicity study was conducted according to the OECD Guidelines for the Testing of Chemicals (OECD, 1998). Eighty rats (40 males and 40 females) aged 6 weeks were used for the study. Each animal was individually caged and allowed to acclimate to the laboratory conditions for 6 days before treatment. IMD (dose levels: 0, 100, 300 or 1,000 mg/kg/day) was administered daily by gavage to Crl:CD(SD) rats (10 males and 10 females for each dose) for 13 weeks. Ten (10), 30 and 100 mg/mL solutions were prepared for the 100, 300 and 1,000 mg/kg dosing groups, respectively. The dose volume was 1.0 mL per 100 g-BW. The general condition of the animals was observed twice a day (before and after dosing) during the administration period, and body weights and food consumption were determined at Days 1 (before grouping), 8, 15, 22, 29, 36, 43, 50, 57, 64, 71, 78, 85, and 90. The animals to be necropsied were weighed before necropsy (Day 91 or 92). A standard safety pharmacology test, the Functional Observational Battery (FOB), was conducted once a week to examine responses on removal from their cages (ease/difficulty of removal, vocalization), conditions when handheld (muscle tone, subnormal temperature, piloerection, soiled fur, unkempt fur, skin color, lacrimation, exophthalmos, pupillary size, salivation), behavior in an arena (air-righting reflex, posture, motor activity, respiration, eyelids, gait, tremor, twitch, tonic convulsion, clonic convulsion, stereotypic behavior, abnormal behavior), sensorimotor reactivity test (approaching contact, tactile response, auditory response, pain response, pupillary reflex), grip strength test (forelimb, hindlimb), locomotor activity test (amount of movement in a 10 min interval, total amount of movement [1-hr activity]). Urinalysis at the 13th week of administration, ophthalmological examinations during the quarantine period (Day -2, -1) and administration period (Day 83) were conducted. For the urinalysis, fresh urine (within 3 hr of urination) and pooled urine (24 hr) were collected. After the completion of the administration period, clinical examinations (examinations of hematology, blood coagulation, blood chemistry and serum protein electrophoresis) and pathological examinations (organ weight measurement, macroscopic and histopathological examinations) were conducted. Blood samples were collected from the abdominal aorta under isoflurane anesthesia after overnight fasting. For pathological examination, all animals were necropsied after blood sampling and euthanized by exsanguination

under isoflurane anesthesia.

Hematological tests consisted of hematocrit (HCT), hemoglobin (HGB), red blood cell count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte ratio/count (Reticulocyte), platelet count (PLT), white blood cell count (WBC), differential leukocyte percentages, neutrophil count (NEUT), lymphocyte count (LYMPH), monocyte count (MONO), eosinophil count (EOSN), basophil count (BASO), and large unstained cell count (LUC) were measured. Hematology was performed using the ADVIA120 system (Bayer AG, Leverkusen, Germany). A STA Compact coagulation analyzer (Roche Diagnostics K.K., Tokyo, Japan) was used to measure blood prothrombin time, active partial thromboplastin time, and fibrinogen. Blood chemistries consisted of total protein (T-Protein), glucose, triglyceride (TG), total cholesterol (T-Cho), blood urea nitrogen (BUN), creatinine, total bilirubin (T-Bilirubin), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP), calcium (Ca), inorganic phosphorous (iP), sodium (Na), potassium (K), and chloride (Cl). Sodium, K, and Cl were measured by an EA06R electrolyte analyzer (A&T Corporation, Kanagawa, Japan). All other blood chemistry values were determined with an automatic multi-analyzer Hitachi 7170 (Hitachi, Ltd., Tokyo, Japan). Serum protein electrophoresis of albumin, α_1-globulin, α_2-globulin, β-globulin, γ-globulin, albumin/total globulin ratio (A/G) were measured using a Epalyzer 2 Plus electrophoresis analyzer (Helena Laboratories, Beaumont, TX, USA). Urinalysis measured pH, occult blood, ketone bodies, glucose, protein, bilirubin, urobilinogen, urinary volume, color, osmotic pressure, sediment, Na, K and Cl. pH, occult blood, ketone bodies, glucose, protein, bilirubin, urobilinogen were measured with either the N-Multistix SG-L (Siemens Healthcare Diagnostics K.K., Tokyo, Japan) or Clinitek 500 (Bayer AG, Leverkusen, Germany). Osmotic pressure was measured by the cryoscopic method. Sediment was measured by microscopic examination. Sodium, K, and Cl were measured using an EA06R electrolyte analyzer.

Human tolerance NOAEL studies for loose stools, and other gastrointestinal symptoms

Volunteer subjects were used in the first study; however, a NOAEL could not be determined. Therefore a second study was conducted.

Study Ⅰ. 40 healthy subjects (age, 39.3 ± 6.9 years of age; female/15, male/25; body height, 166.1 ± 9.0 cm; BW, 58.1 ± 9.7 kg) were enrolled in a dose escalation study. The first dose was administered as a single- blind crossover study in which 30 g of either IMD or maltodextrin (MD; Pinedex #1, Matsutani Chemical Industry Co., Ltd., Hyogo, Japan) was administered in 200 mL water. The carbohydrates were given 2 hr after consuming a normal meal. Escalating treatments were ingested in a similar manner with 200 mL water solutions containing IMD at 40, 50, 60, and 70 g. There was at least a 3-day rest between treatments. No additional MD was consumed. The gastrointestinal reaction of the subjects to the ingestion of the various doses of IMD, and MD used as a control, were recorded by the test subjects. The tolerance-related items included: the time from consumption till the first bowel movement, shape/consistency of feces during the first 24 hr after consumption, change in the smell of feces, and abdominal symptoms (upper abdominal pain, lower abdominal pain, tenesmus, borborygmus, abdominal bloating, flatulence, weakness of low back, vomiting, discomfort, nausea, and other). The subjects were also asked to provide any other specific comments, if they desired. The status of feces was graded as: "very hard," "banana-shaped," "soft," "muddy," and "watery." In these studies the term loose stools is defined as the condition when the feces were reported to be "muddy" or "watery."

Study Ⅱ. A total of 20 healthy subjects (age, 41.8 ± 10.4 years; female/10, male/10; body height, 162.8 ± 7.7 cm; BW, 54.4 ± 8.5 kg; BMI, 20.5 ± 2.4 kg/m^2) who had at least 3 defecations per week were enrolled in a second dose escalation study. Based on the results of the study Ⅰ (see Results below), the beginning dose in study Ⅱ was decided to be 0.8 g/kg-BW, which was unlikely to cause loose stools. The first dose was administered as a single-blind crossover study in which 0.8 g/kg-bw of either IMD or MD was administered in 300 mL water. Additional treatments were ingested in a similar manner with solutions containing IMD at 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2 and 2.4 g/kg, in 300 mL water. The other study conditions were similar to those in the study Ⅰ.

Four-week high-dose ingestion study in humans

The third study was not blinded or placebo-controlled, and was conducted at the Miura Clinic (Osaka, Japan). Healthy men and women (age ≥ 20 and ≤ 64 years) were recruited. Thirty volunteers visited the clinic for preliminary examinations including medical interviews, physical examinations, and laboratory tests. A total of 20 subjects (age 42.6 ± 11.2 years; female/11, male/9) who were considered suitable for the study by the inclusion/exclusion criteria were enrolled. IMD was provided to each of the 20 subjects, and 30 g (dsb) of IMD were consumed

once daily for 4 weeks. The intake time and method were *ad libitum*. They visited the clinic at 2 and 4 weeks after the start of intake, and at 2 weeks after the completion of intake. The subjects received medical interviews, physical measurements, physical examinations and laboratory tests. The subjects recorded their daily intake of IMD, amount of alcohol consumption, and amount of exercise in a diary every day from the start to the end of IMD intake. Height, body weight, systolic and diastolic blood pressure (SBP, DBP), and pulse rate were measured in the physical examination. The following laboratory tests were performed: hematological tests (WBC, RBC, HGB, HCT, MCV, MCH, MCHC, and PLT); blood chemistry tests (uric acid, urea nitrogen, AST, ALT, γ-GTP, ALP, lactic acid dehydrogenase [LDH], T-Bilirubin, T-Protein, albumin, creatinine, creatine phosphokinase [CPK], serum amylase, T-Cho, HDL-Cho, LDL-Cho, TG, glucose, HbA1c, Na, Cl, K, Mg, Ca, Fe, and iP); and urine analysis (protein, urinary sugar, urobilinogen, bilirubin, occult blood, ketone bodies, specific gravity, pH, creatinine). The following methods were used: *Hematological tests*—electric resistance detection method (RBC, MCV, MCH, MCHC, and PLT), flow cytometry (WBC), SLS-Hb method (HGB), and red blood cell pulse height detection method (HCT); *Blood chemistry tests*—enzymatic assays (uric acid, T-Bilirubin, creatinine, serum amylase, T-Cho, HDL-Cho, LDL-Cho, TG, glucose, HbA1c, and iP), Japan Society of Clinical Chemistry (JSCC) transferable method (AST, ALT, γ-GTP, ALP, LDH, and CPK), electrode method (Na, K, Cl, Mg), biuret method (T-Protein), urease-LEDH method (urea nitrogen), arsenazo III method (Ca), colorimetric method (Fe), and the improved BCP method (albumin); and *Urine analysis*—enzymatic method (creatinine), refractometer method (specific gravity), and the test-strip method (other test items).

Twelve-week low-dose ingestion study in humans

A second non-blinded, non-placebo controlled study was conducted at the Miura Clinic (Osaka, Japan). Healthy men and women (age ≥ 20 and ≤ 64 years) were recruited. Thirty volunteers received preliminary examinations including medical interviews, physical examinations, and laboratory tests. A total of 20 subjects who met the inclusion/exclusion criteria were enrolled. Eighty four (84) 10 g packets of IMD was given to each of the 20 subjects. The subjects consumed 10 g (dsb) once daily for 12 weeks. One subject dropped out voluntarily due to personal circumstances after the start of the study. A total of 19 subjects excluding the one dropout (age 44.2 ± 11.2 years; female/12, male/7) were enrolled. The intake time and method were *ad libitum*. Subjects visited the clinic at 4, 8 and 12 weeks after the start of intake and at week 4 after completion of intake. Subjects received medical interviews, physical examinations, physiological tests and laboratory tests. The subjects recorded their daily intake of IMD, amount of alcohol consumption, and amount of exercise in a diary every day from the start to the end of IMD intake.

The test items and methods were the same as those performed in the 4-week high-dose ingestion study.

Statistical analysis

All values were expressed as the mean ± S.D. Statistical analyses were performed by the following methods.

Micronucleus test: If the data were not within the acceptable range, they were analyzed by a Conditional Binomial test (Kastenbaum and Bowman method: upper tailed significance level of 0.025) between the negative control group and each test substance group. In the positive control, the data on the frequency of MNIE as compared to that in the negative control group was analyzed by the Conditional Binomial test (upper tailed significance level of 0.025).

Chromosome aberration test: The incidence of aberrant cells was analyzed by Fisher's exact test (one-sided significance level 2.5%). If significant differences were observed in the test substance groups, concentration dependency was analyzed by the Cochran-Armitage trend test (one-sided significance level 2.5%).

90-day repeated-oral dose toxicity study in rats: The quantitative data were initially analyzed by Bartlett's test for equality of variance at a two-sided significance level of 20%. When Bartlett's test showed homoscedasticity, the data were analyzed by Dunnett's multiple comparison test based on the exact probability calculation for imbalanced data. When the Bartlett's test showed heteroscedasticity, the data were analyzed by the Dunnett's fashion test with Satterthwaite approximation and step down. In both of these analyses, the significance level vs. the control group was at a two-sided 5% or 1%. The FOB data were analyzed by Steel's test at a two-sided 5% or 1%. Data from the examinations for ophthalmology and histopathology were analyzed by the paired comparisons based on the step-down permutated multiplicity adjustment calculated by Fisher's exact test at a two-sided significance level of 5% and 1%.

Four-week high-dose and 12-week low-dose ingestion studies in humans: The changes from the baseline (week 0) in body weight, BMI, SBP, DBP, pulse, hematological tests, blood chemistry tests, and urine analysis were analyzed by paired t-test with a Bonferroni correction. In

the urine dipstick test, the scores of -, ±, +, ++, and +++ were converted to 0, 1, 2, 3, and 4, respectively, and the data were analyzed using Wilcoxon signed-rank test with Bonferroni correction. The significance level was set at $p < 0.05$ for both analyses.

RESULTS

Bacterial reverse mutation test

Based on the results of the 1st and 2nd main studies, the mean number of revertant colonies in all bacterial strains was less than twice the negative control values at all doses of IMD, regardless of the absence or presence of S9 mix, without dose dependency. In the positive control group the number of revertant colonies in all strains was markedly increased when compared to that of the negative control group. Growth inhibition by IMD and solubility issues were not observed at any dose, or with any bacterial strain in either the absence or presence of S9 mix.

Micronucleus test

No difference in body weight gain, or abnormal clinical signs were observed in any of the groups treated with IMD. In the groups treated with 500, 1,000 and 2,000 mg/kg of IMD, the frequencies of MNIE were 0.20%, 0.07% and 0.12%, respectively. All of these were within the acceptable ranges (mean ± 3 S.D.) calculated from the historical data of the test laboratory. The ratios of IE to the total number of analyzed erythrocytes, which are indexes of the influence of the test substance on the bone marrow cells, were 57.0, 59.3 and 60.7% in the 500, 1,000 and 2,000 mg/kg groups, respectively. In the negative control group, 0 to 4 MINIE in 2,000 IE per animal were observed and the group mean frequency was 0.11% (0-0.2%). The ratio of IE to the total number of analyzed erythrocytes was 57.0% in the negative control group. No statistically significant decreases in the ratio of IE were observed in any of the IMD-treated groups as compared with that in the negative control group. In the positive control group treated intravenously with MMC at a dose of 2 mg/kg, the frequency of MNIE (3.03%) was significantly increased ($p \leq 0.025$) as compared with that in the negative control group, and the ratio of IE was 44.0%.

Chromosome aberration test

In the -S9 assay, the incidences of cells with structural chromosome aberrations in the groups treated with IMD at 1,250, 2,500 and 5,000 µg/mL were 2.5, 0.5 and 1.0%, respectively. These values were not statistically significant in comparison with the incidence in the negative control group (0.5%). The incidences of polyploid cells in the groups treated with IMD at 1,250, 2,500 and 5,000 µg/mL were 0.0, 0.0 and 1.0%, respectively, which were not statistically different from the negative control group (0.5%). Cell growth inhibition was not observed at any concentration. At the start and end of the treatments, precipitation was not observed at any concentration. Conversely, there was a high incidence of cells with structural chromosomal aberrations (63.0%) in the positive control group, which was significantly higher than the negative control group (0.5%). In the +S9 assay group, the incidences of cells with structural chromosome aberrations treated with IMD at 1,250, 2,500 and 5,000 µg/mL were 0.0, 1.0 and 1.5%, respectively. These percentages were not statistically different from the negative control group (0.5%). There were no polyploid cells in the groups treated with IMD at 1,250, 2,500 and 5,000 µg/mL, which was the same as the negative control group (0.0%). Neither cell growth inhibition nor precipitation was observed at any concentration at either the start or end of the study period. As expected there was a high incidence of cells with structural chromosomal aberrations (65.0%) in the positive control group, which was statistically greater than that in the negative control group (0.5%). In the 24-hr assay, the incidences of cells with structural chromosome aberrations or polypliod cells in the groups treated with IMD at 1,250, 2,500 and 5,000 µg/mL were 0.5, 1.0 and 0.5%, and 0.0, 0.0 and 0.0%, respectively. None of the comparisons to the incidences in the negative control group (0.0%) were significantly different. Cell growth inhibition was not observed at any concentration, and no precipitation was observed at any time or concentration. The positive control group had a significantly higher percentage of structural chromosomal aberrations (49.0%) than the negative control group (0.0%). The results of this study revealed no significant increase in the incidence of cells with structural chromosomal aberrations or polyploid cells when treated with IMD in any of the assay conditions. Both MMC and CP, as the positive controls for the -S9 and 24-hr assays and the +S9 assay, respectively, significantly induced structural chromosomal aberrations.

Acute oral toxicity study in rats

All 5 animals survived and no animals showed abnormal clinical signs during the observation period. The rats showed normal body weight gains during the observation period and showed no abnormal gross findings in the examined organs and tissues.

90-day repeated-oral dose toxicity study in rats

The results of hematology, blood chemistry, urine

analysis, and tissue weight per body weight are shown in Tables 2, 3, 4, and 5, respectively. Observation of the animals showed no deaths in either the males or females in any group throughout the administration period. Furthermore, there were no adverse effects due to the test substance on clinical signs, body weight, body weight gain, food consumption, blood coagulation, blood chemistry, ophthalmology, organ weight, and histopathological findings. Review of the hematologic data showed that the hemoglobin concentration in the 1,000 mg/kg/day administered male group was significantly lower than that in the male control group. However, this change was judged not to be toxicologically significant because the change was very slight, within the normal range and there were no changes in other parameters related to erythrocytes (hematocrit, mean corpuscular volume, etc.). The ratio of monocytes in the 300 mg/kg/day administered female group was significantly less than in the female control group. This difference was not dose dependent, and within the normal range for this variable. The concentration of α-2 globulin in the 1,000 mg/kg/day male group was significantly higher than that in the male control group, but was still within the normal range. Evaluation of the urinalysis values showed that the total sodium, potassium and chloride excretion in the 1,000 mg/kg/day female group and the total sodium excretion in the 300 mg/kg/day female group were significantly lower than those in the female control group. However, all of the significantly lower values were not outside the normal range for these animals. Additionally there were no significant differences in the serum ion concentrations (sodium, potassium and chloride).

Human tolerance studies (No observed adverse effect level for loose stools, and other gastrointestinal symptoms)

Study I. None of the subjects consuming the 30 g MD reported loose stools (muddy or watery). 35 of the 40 subjects that ingested the MD did not report any abdominal symptoms. The remaining 5 subjects reported a total of 4 different symptoms (Table 6).

The occurrence of loose stools was not observed with IMD treatment except in one subject administered the 60-g dose, and in a second subject who ingested the 70-g dose. Upon interviewing the subject who reacted to the 60-g dose, it was found that the subject had developed

Table 2. Hematology data from the 90-day repeated-oral dose toxicity study in rats administered IMD.

Sex		Male				Female			
Dose (mg/kg/day)		0	100	300	1000	0	100	300	1000
Number of animals		10	10	10	10	10	10	10	10
HCT (%)		45.5 ± 1.9	44.6 ± 1.9	45.5 ± 1.3	44.1 ± 0.7	43.8 ± 1.7	43.5 ± 2.8	43.6 ± 1.7	43.3 ± 1.3
HGB (g/dL)		15.6 ± 0.7	15.2 ± 0.6	15.5 ± 0.4	15.0 ± 0.3#	15.1 ± 0.5	15.1 ± 1.0	15.0 ± 0.5	14.8 ± 0.3
RBC ($\times 10^6$/mm^3)		8.60 ± 0.42	8.45 ± 0.39	8.61 ± 0.30	8.49 ± 0.32	8.14 ± 0.52	8.06 ± 0.63	8.03 ± 0.37	7.90 ± 0.39
MCV (μm^3)		53.0 ± 1.8	52.9 ± 1.8	52.9 ± 1.9	52.1 ± 1.6	53.9 ± 1.5	54.1 ± 1.5	54.4 ± 1.2	54.9 ± 1.9
MCH (pg)		18.2 ± 0.7	18.0 ± 0.7	18.0 ± 0.7	17.7 ± 0.6	18.5 ± 0.6	18.7 ± 0.6	18.7 ± 0.6	18.8 ± 0.8
MCHC (%)		34.3 ± 0.6	34.1 ± 0.5	34.0 ± 0.2	34.0 ± 0.5	34.4 ± 0.4	34.6 ± 0.6	34.5 ± 0.5	34.2 ± 0.4
Reticulocyte (%)		2.2 ± 0.6	2.2 ± 0.3	2.1 ± 0.2	2.0 ± 0.3	2.0 ± 0.2	1.8 ± 0.3	2.2 ± 0.3	2.2 ± 0.6
($\times 10^9$/L)		189.5 ± 45.5	181.7 ± 20.2	183.9 ± 23.6	168.9 ± 29.9	159.6 ± 13.7	141.9 ± 19.9	173.2 ± 20.9	171.3 ± 42.3
PLT ($\times 10^3$/mm^3)		1069 ± 282	1000 ± 96	979 ± 75	1015 ± 75	995 ± 120	922 ± 94	982 ± 107	997 ± 115
WBC ($\times 10^3$/mm^3)		9.27 ± 2.53	8.34 ± 1.79	8.38 ± 2.05	7.11 ± 1.97	5.06 ± 1.62	4.76 ± 1.05	4.79 ± 1.13	5.52 ± 1.41
Differential leukocyte ratios (%)	NEUT	17.4 ± 7.4	17.5 ± 4.6	18.4 ± 5.6	16.8 ± 3.8	18.2 ± 7.8	16.8 ± 4.7	16.0 ± 3.3	14.5 ± 5.7
	LYMPH	77.3 ± 7.8	76.5 ± 6.2	76.0 ± 6.1	77.2 ± 4.9	75.6 ± 8.4	77.0 ± 5.1	78.4 ± 3.4	80.1 ± 5.7
	MONO	2.6 ± 0.8	3.3 ± 1.3	2.9 ± 0.8	3.2 ± 1.1	3.1 ± 0.8	2.9 ± 0.8	2.2 ± 0.5*	2.8 ± 0.8
	EOSN	1.8 ± 0.6	1.7 ± 0.5	1.8 ± 0.8	1.8 ± 0.6	2.4 ± 1.0	2.4 ± 0.9	2.6 ± 0.8	1.7 ± 0.6
	BASO	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0
	LUC	0.8 ± 0.3	0.9 ± 0.4	0.8 ± 0.4	0.9 ± 0.4	0.6 ± 0.1	0.9 ± 0.3	0.7 ± 0.1	0.8 ± 0.4
NEUT ($\times 10^3$/mm^3)		1.62 ± 0.84	1.43 ± 0.38	1.48 ± 0.37	1.19 ± 0.42	0.89 ± 0.43	0.78 ± 0.23	0.76 ± 0.23	0.77 ± 0.31
LYMPH ($\times 10^3$/mm^3)		7.16 ± 2.07	6.42 ± 1.65	6.43 ± 1.83	5.50 ± 1.61	3.86 ± 1.43	3.68 ± 0.93	3.76 ± 0.93	4.44 ± 1.21
MONO ($\times 10^3$/mm^3)		0.24 ± 0.10	0.27 ± 0.12	0.24 ± 0.10	0.23 ± 0.08	0.16 ± 0.06	0.14 ± 0.05	0.10 ± 0.02	0.16 ± 0.07
EOSN ($\times 10^3$/mm^3)		0.16 ± 0.05	0.14 ± 0.03	0.15 ± 0.07	0.12 ± 0.04	0.12 ± 0.05	0.11 ± 0.05	0.13 ± 0.05	0.09 ± 0.04
BASO ($\times 10^3$/mm^3)		0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.01	0.00 ± 0.00	0.01 ± 0.00
LUC ($\times 10^3$/mm^3)		0.08 ± 0.04	0.07 ± 0.04	0.06 ± 0.04	0.07 ± 0.04	0.03 ± 0.01	0.04 ± 0.02	0.03 ± 0.01	0.05 ± 0.03

Values are mean ± S.D. *Significantly different from the control using Dunnett's test at $p < 0.05$. #Significantly different from the control using Dunnett fashion test with Satterthwaite approximation and step down at $p < 0.05$.

Table 3. Blood chemistry and serum protein electrophoresis data from the 90-day repeated-oral dose toxicity study in rats administered IMD.

Sex	Male				Female			
Dose (mg/kg/day)	0	100	300	1000	0	100	300	1000
Number of animals	10	10	10	10	10	10	10	10
T. protein (g/dL)	5.88 ± 0.24	5.92 ± 0.25	6.00 ± 0.17	5.83 ± 0.31	6.32 ± 0.39	6.15 ± 0.30	6.49 ± 0.49	6.39 ± 0.46
Glucose (mg/dL)	153 ± 16	164 ± 20	167 ± 33	164 ± 24	142 ± 21	147 ± 22	146 ± 16	144 ± 15
Triglyceride (mg/dL)	62.2 ± 13.7	80.8 ± 52.7	69.1 ± 25.1	64.6 ± 39.5	28.8 ± 21.5	25.2 ± 10.8	25.0 ± 19.8	35.6 ± 34.6
T. Cho (mg/dL)	64 ± 6	75 ± 14	77 ± 14	73 ± 14	83 ± 14	80 ± 19	85 ± 18	80 ± 22
BUN (mg/dL)	13.2 ± 1.8	13.3 ± 1.7	14.0 ± 2.3	13.6 ± 1.9	16.5 ± 3.2	17.0 ± 2.4	14.7 ± 3.0	15.8 ± 1.4
Creatinine (mg/dL)	0.28 ± 0.04	0.29 ± 0.03	0.30 ± 0.03	0.30 ± 0.03	0.37 ± 0.08	0.38 ± 0.08	0.29 ± 0.04#	0.31 ± 0.04
T. Bilirubin (mg/dL)	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.09 ± 0.02	0.08 ± 0.02	0.08 ± 0.02	0.10 ± 0.02
AST (U/L)	76 ± 12	86 ± 22	76 ± 13	72 ± 12	81 ± 16	77 ± 11	95 ± 29	80 ± 16
ALT (U/L)	28 ± 5	32 ± 8	28 ± 4	27 ± 5	24 ± 6	23 ± 6	32 ± 15	30 ± 10
ALP (U/L) SY	307 ± 52	300 ± 36	334 ± 91	282 ± 67	175 ± 51	157 ± 32	158 ± 30	154 ± 53
γ-GTP (U/L)	0.3 ± 0.2	0.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.2	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.2	0.4 ± 0.3
Ca (mg/dL)	9.64 ± 0.21	9.77 ± 0.10	9.75 ± 0.27	9.68 ± 0.26	9.76 ± 0.37	9.72 ± 0.29	9.79 ± 0.64	9.90 ± 0.41
iP (mg/dL)	5.83 ± 0.32	5.88 ± 0.56	5.61 ± 0.57	5.48 ± 0.69	4.91 ± 0.93	5.22 ± 0.73	5.35 ± 0.95	5.51 ± 0.93
Na (mmol/L)	142.9 ± 1.0	142.2 ± 1.4	142.0 ± 1.3	141.8 ± 1.1	141.9 ± 1.0	141.7 ± 1.2	142.1 ± 0.9	142.1 ± 0.7
K (mmol/L)	4.66 ± 0.33	4.71 ± 0.25	4.68 ± 0.24	4.70 ± 0.14	4.37 ± 0.14	4.17 ± 0.35	4.21 ± 0.14	4.28 ± 0.29
Cl (mmol/L)	106.5 ± 1.1	105.7 ± 0.6	105.5 ± 1.5	106.3 ± 0.9	108.4 ± 1.5	107.2 ± 1.1	108.2 ± 2.0	107.0 ± 0.8
Serum protein electrophoresis								
Albumin (%)	49.7 ± 2.0	48.6 ± 2.7	49.5 ± 2.7	49.8 ± 2.3	57.0 ± 2.7	57.4 ± 2.7	59.3 ± 2.8	57.7 ± 2.2
α_1-globulin (%)	21.1 ± 2.7	21.2 ± 3.1	20.8 ± 3.2	20.7 ± 2.8	15.6 ± 2.9	15.7 ± 2.8	14.6 ± 2.7	15.8 ± 2.0
α_2-globulin (%)	7.9 ± 0.7	8.0 ± 0.9	8.2 ± 0.9	8.9 ± 1.1	6.7 ± 0.9	7.2 ± 1.1	6.6 ± 0.8	6.7 ± 1.1
β-globulin (%)	16.1 ± 0.7	16.7 ± 1.0	16.6 ± 0.9	16.4 ± 1.3	14.6 ± 1.1	14.4 ± 1.6	14.3 ± 1.1	14.2 ± 1.2
γ-globulin (%)	5.3 ± 1.7	5.4 ± 2.2	4.9 ± 1.5	4.3 ± 0.5	6.1 ± 1.6	5.4 ± 1.4	5.2 ± 1.2	5.6 ± 0.6
A/G	0.99 ± 0.08	0.95 ± 0.10	0.99 ± 0.10	1.00 ± 0.09	1.34 ± 0.15	1.35 ± 0.15	1.47 ± 0.17	1.37 ± 0.12
Albumin (g/dL)	2.92 ± 0.11	2.87 ± 0.12	2.97 ± 0.16	2.89 ± 0.08	3.61 ± 0.34	3.53 ± 0.28	3.85 ± 0.39	3.69 ± 0.39
α_1-globulin (g/dL)	1.24 ± 0.18	1.26 ± 0.22	1.25 ± 0.21	1.21 ± 0.22	0.99 ± 0.20	0.97 ± 0.19	0.95 ± 0.21	1.01 ± 0.15
α_2-globulin (g/dL)	0.46 ± 0.04	0.47 ± 0.05	0.49 ± 0.05	0.51 ± 0.05*	0.42 ± 0.05	0.44 ± 0.07	0.42 ± 0.04	0.43 ± 0.06
β-globulin (g/dL)	0.95 ± 0.05	0.99 ± 0.08	0.99 ± 0.06	0.96 ± 0.10	0.92 ± 0.07	0.88 ± 0.09	0.92 ± 0.06	0.91 ± 0.06
γ-globulin (g/dL)	0.31 ± 0.11	0.32 ± 0.14	0.30 ± 0.09	0.25 ± 0.04	0.38 ± 0.10	0.33 ± 0.08	0.34 ± 0.08	0.36 ± 0.04

Values are mean ± S.D. *Significantly different from the control using Dunnett's test at $p < 0.05$. #Significantly different from the control using Dunnett fashion test with Satterthwaite approximation and step down at $p < 0.05$.

symptoms and signs of a common cold, and experienced loose stools following IMD consumption. It was thought likely that the cold might have been the cause of the loose stools. The subject proceeded to the next higher dose of IMD, and after consuming the 70-g dose reported no loose stools in the subsequent 24 hr. The study staff concluded that the loose stools after the 60-g dose was not likely the cause of the IMD. Therefore, the reported loose stools after consuming the 60 g of IMD was not recorded as an adverse event. The subject who reported loose stools after ingesting 70 g of IMD reported the feces as "muddy." Five subjects reported "soft" feces after taking the 30-g dose, 4 at the 40-g dose, and 9 at ≥ 50-g doses, suggesting a tendency of soft stools at higher doses. There was no difference in the timing of the first bowel movement among the doses, so IMD did not appear to have an effect on bowel emptying. The number of subjects not reporting any abdominal effects in the 30-70 g treatment groups were, 35, 34, 27, 26, and 27, respectively. Further, the number of subjects reporting abdominal symptoms, and the total number of symptoms reported at each dose were, 5/5, 6/6, 13/19, 14/21, and 13/19, respectively for an average of 1.4 symptoms per subject that reported symptoms. These data suggest minimal symptoms below the 50-g dose, with a stable increase in the number of symptoms and subjects of about 2.5 times at 50 g IMD or greater. Of the total 70 abdominal complaints, the majority (52; 74%) were reported as borborygmus, abdominal bloating and flatus. Two symptoms reported in the MD control group were similar to the

Table 4. Urinalysis data from the 90-day repeated-oral dose toxicity study in rats administered IMD.

Sex		Male				Female			
Dose (mg/kg/day)		0	100	300	1000	0	100	300	1000
Number of animals		10	10	10	10 (9)	10	10	10	10
Volume (mL)		16.3 ± 6.6	17.4 ± 5.8	20.1 ± 9.4	18.9 ± 10.6	16.5 ± 7.1	14.1 ± 8.4	12.2 ± 6.0	14.3 ± 6.2
Osmotic pressure (mOsm/kg)		1279 ± 499	1323 ± 449	1214 ± 499	1075 ± 399	1103 ± 393	1395 ± 724	1315 ± 481	1027 ± 458
Na (mmol/L)		51.7 ± 33.8	60.9 ± 27.3	53.1 ± 17.1	37.7 ± 22.9	75.4 ± 26.2	93.7 ± 67.0	74.7 ± 36.7	61.2 ± 31.4
K (mmol/L)		165.2 ± 70.8	171.9 ± 61.9	150.9 ± 62.3	132.3 ± 54.8	152.9 ± 56.0	193.9 ± 116.7	173.5 ± 62.9	131.3 ± 55.9
Cl (mmol/L)		78.7 ± 50.1	75.5 ± 38.5	68.6 ± 25.6	55.0 ± 31.3	98.7 ± 40.1	126.9 ± 89.3	102.8 ± 49.8	75.4 ± 34.9
Total excretion (mmol/day)									
Na		0.70 ± 0.25	1.03 ± 0.48	1.02 ± 0.49	0.62 ± 0.29	1.11 ± 0.24	0.99 ± 0.28	0.79 ± 0.30*	0.78 ± 0.31*
K		2.35 ± 0.47	2.75 ± 0.58	2.62 ± 0.72	2.05 ± 0.53	2.23 ± 0.48	2.07 ± 0.54	1.84 ± 0.45	1.61 ± 0.38*
Cl		1.07 ± 0.41	1.23 ± 0.60	1.31 ± 0.67	0.81 ± 0.39	1.42 ± 0.32	1.33 ± 0.36	1.08 ± 0.37	0.94 ± 0.35*
Color	SY	10	10	10	10	10	9	10	10
	YB	0	0	0	0	0	1	0	0
pH	6.5	0	0	0	0	0	0	0	2
	7.0	0	0	0	1	0	1	0	2
	7.5	1	0	0	0	3	1	1	1
	8.0	0	0	1	1	2	0	5	2
	8.5	4	5	6	5	4	3	4	2
	≥9.0	5	5	3	3	1	5	0	1
Occult Blood	-	7	9	7	5	10	10	10	9
	+/-	1	1	3	5	0	0	0	1
	1+	1	0	0	0	0	0	0	0
	2+	1	0	0	0	0	0	0	0
Ketone Bodies	-	1	6	7	4	10	8	10	9
	+/-	5	2	3	4	0	2	0	1
	1+	4	2	0	2	0	0	0	0
Glucose	-	10	10	10	10	10	10	10	10
Protein (mg/dL)	-	3	2	2	3	10	6	10	9
	+/-	1	4	6	3	0	2	0	1
	30	5	3	2	4	0	2	0	0
	100	1	1	0	0	0	0	0	0
Bilirubin	-	10	10	10	10	10	10	10	10
Urobilinogen (E.U./dL)	0.1	8	9	10	9	9	8	9	9
	1.0	2	1	0	1	1	2	1	1
Erythrocytes	-	10	10	10	10	10	10	10	10
Leukocytes	-	10	10	10	10	10	10	10	10
Squamous Cells	-	10	10	10	10	10	10	10	10
Transitional epithelial cells	-	10	10	10	10	10	10	10	10
Renal tubular epithelial cells	-	10	10	10	10	10	10	10	10
Casts	-	10	10	10	10	10	10	10	10
Fat globules	-	10	10	10	10	10	10	10	10
Mucous threads	-	8	10	9	9	10	10	10	9
	+	2	0	1	1	0	0	0	1
Crystals	-	0	1	0	4	0	0	0	0
	+	10	9	10	6	10	10	10	10

Values are mean ± S.D. *Significantly different from the control using Dunnett's test at $p < 0.05$ in volume, osmotic pressure and electrolytes. Number in parentheses expresses the number of animals in the chloride. Color: SY, Slight yellow; YB, Yellow-brown. Occult Blood: -, Negative; +/-, 0.015 mg/dL; 1+, 0.062 mg/dL; 2+, 0.135 mg/dL. Ketone bodies: -, Negative; +/-, 5 mg/dL; 1+, 15 mg/dL. Glucose: -, Negative. Protein: -, Negative; +/-, 15 mg/dL. Bilirubin: -, Negative. Erythrocytes: -, Negative. Leukocytes: -, Negative. Squamous Cells: -, Negative. Transitional epi. : -, Negative. Renal tubular epi. : -, Negative. Casts: -, Negative. Fat globules: -, Negative. Mucous threads: -, Negative; +, Positive. Crystals: -, Negative; +, Positive.

Table 5. Relative organ weight data from the 90-day repeated-oral dose toxicity study in rats administered IMD.

Sex	Male				Female			
Dose (mg/kg/day)	0	100	300	1000	0	100	300	1000
Number of animals	10	10	10	10	10	10	10	10
Body weight (g)	522 ± 47	579 ± 55	579 ± 39	589 ± 57	283 ± 34	287 ± 43	267 ± 27	287 ± 21
Brain (%)	0.417 ± 0.031	0.406 ± 0.041	0.402 ± 0.039	0.401 ± 0.035	0.729 ± 0.079	0.720 ± 0.080	0.764 ± 0.048	0.730 ± 0.056
Heart (%)	0.281 ± 0.011	0.286 ± 0.026	0.290 ± 0.022	0.277 ± 0.021	0.335 ± 0.030	0.326 ± 0.021	0.330 ± 0.026	0.323 ± 0.019
Lungs (%)	0.289 ± 0.017	0.286 ± 0.018	0.286 ± 0.016	0.275 ± 0.020	0.436 ± 0.031	0.421 ± 0.020	0.418 ± 0.023	0.408 ± 0.029
Liver (%)	2.493 ± 0.111	2.696 ± 0.209#	2.604 ± 0.081	2.585 ± 0.223	2.427 ± 0.106	2.444 ± 0.169	2.508 ± 0.170	2.440 ± 0.149
Kidneys (%)	0.610 ± 0.048	0.620 ± 0.039	0.596 ± 0.045	0.572 ± 0.031	0.653 ± 0.040	0.669 ± 0.042	0.675 ± 0.051	0.666 ± 0.052
Spleen (%)	0.144 ± 0.023	0.147 ± 0.018	0.150 ± 0.025	0.142 ± 0.019	0.186 ± 0.037	0.177 ± 0.032	0.182 ± 0.011	0.172 ± 0.023
Adrenal glands (%)	0.012 ± 0.002	0.011 ± 0.001	0.011 ± 0.001	0.011 ± 0.003	0.023 ± 0.004	0.026 ± 0.004	0.024 ± 0.003	0.023 ± 0.003
Testes (%)	0.632 ± 0.052	0.623 ± 0.051	0.633 ± 0.059	0.619 ± 0.071	-	-	-	-
Ovaries (%)	-	-	-	-	0.030 ± 0.007	0.030 ± 0.008	0.031 ± 0.005	0.029 ± 0.006

Values are mean ± S.D. #Significantly different from the control using Dunnett fashion test with Satterthwaite approximation and step down at $p < 0.05$.

Table 6. Occurrence of abdominal symptoms (multiple answers allowed) in study Ⅰ.

		MD	IMD				
			30 g	40 g	50 g	60 g	70 g
Abdominal symptoms	Without symptoms	35	35	34	27	26	27
	Upper abdominal pain	0	0	1	1	1	1
	Lower abdominal pain	1	0	0	0	0	0
	Tenesmus	1	1	1	1	1	2
	Borborygmus	0	1	0	6	4	5
	Abdominal bloating	0	1	4	4	7	6
	Flatus	1	0	0	5	5	4
	Weakness in low back	0	0	0	0	0	0
	Vomiting	0	0	0	0	0	0
	Discomfort	2	2	0	2	2	1
	Nausea	0	0	0	0	0	0
	Other	0	0	0	0	1*	0

*a feeling of IMD lying heavy on the stomach

number reported at all doses of the IMD group, namely tenesmus, and "discomfort".

IMD ingestion per kilogram BW was calculated by dividing the loose stool-causing dose by the BW. The cumulative incidence of loose stools was obtained by dividing the cumulative number of subjects with loose stools by the total number of subjects provided with each dose. A simple linear regression analysis between IMD ingestion per kilogram BW and the cumulative incidence of loose stools was to be performed, and a NOAEL for loose stools was to be calculated according to the method of Oku *et al*. (Oku and Okazaki, 1998). However, as only one subject was considered to have loose stools as a result of consumption of IMD, the NOAEL for loose stools could not be calculated by this method.

Study Ⅱ. Table 7 shows the occurrence of loose stools and abdominal symptoms on a gram per kg basis. One female subject complained of a transient poor physical condition after consuming the 1.8 g/kg-BW dose. This was considered unlikely to be associated with the study by the investigator, but the subject stopped participating in the study thereafter as a precaution. The subject's data were excluded from the analysis. No subjects developed loose stools in this cross-over study with IMD and MD at the minimum dose of 0.8 g/kg-BW. Loose stools were reported by two subjects (female and male) at a dose of 1.0 g/kg-BW, by one subject (male) at 2.0 g/kg-BW, and by one subject (male) at 2.2 g/kg-BW. The stools were "muddy" in these subjects. These subjects continued to consume increasing doses of IMD. As observed in this group of 4 subjects, loose stools were not consistently observed with increasing doses, and the stool characteristics did not significantly change with increasing doses. Regarding abdominal symptoms, the number of asymp-

Table 7. Occurrence of loose stools and abdominal symptoms in study Ⅱ.

		MD	IMD								
			0.8 g	1.0 g	1.2 g	1.4 g	1.6 g	1.8 g	2.0 g	2.2 g	2.4 g
Loose stools		0	0	2	0	0	0	0	1	1	0
Abdominal symptoms*	Without symptoms	18	17	17	17	15	17	16	12	11	10
	Upper abdominal pain	0	0	0	1	0	0	0	0	0	0
	Lower abdominal pain	0	0	0	0	0	0	0	0	0	0
	Tenesmus	0	0	0	0	0	1	0	1	0	0
	Borborygmus	1	1	1	0	0	0	0	1	2	1
	Abdominal bloating	1	1	1	0	0	1	1	3	4	4
	Flatus	0	0	0	0	0	1	1	2	0	1
	Weakness in low back	0	0	0	0	1	0	0	0	0	0
	Vomiting	0	0	0	0	0	0	0	0	0	0
	Discomfort	0	0	1	0	0	0	0	0	0	0
	Nausea	0	0	0	0	0	0	0	0	0	0
	Other	0	1	0	0	2	0	1	0	0	0

* multiple answers allowed

tomatic subjects at each dose (increasing) was 17, 17, 17, 15, 17, 16, 12, 11 and 10, respectively. Most of the increase in abdominal complaints was at a dose of 2.0 g/kg-BW or higher was because of “bloating”. “Other” (Table 7) abdominal complaints included a feeling of having loose stools without defecation, “mild heart burn,” and a “feeling of retention in the abdomen.” The symptoms of subjects who developed loose stools were transient, and there were no significant health problems reported thereafter. The abdominal symptoms of subjects were also transient.

Four-week high-dose ingestion study in humans

The results of physiological tests, hematological tests, blood chemistry tests, and urine analysis are shown in Tables 8, 9, 10, and 11, respectively. There were no dropouts or discontinuations reported in this study, and 20 subjects were included. The intake compliance rate of IMD was 98.8 ± 3.5%. There were significant differences ($p < 0.05$) in BW and BMI at week-2, and 2 weeks after consumption, but not at 4 weeks as compared to the week 0 values. The increases in the mean weights at those sampling times were 0.55 and 0.67 kg, respectively (Table 8). After 2 weeks of IMD consumption the WBC mean value was significantly lower ($p < 0.05$) than the week 0 value. Likewise the HCT for females and all subjects at 2 weeks of consumption was less than week 0, and the MCH and MCHC for all subjects at weeks 2 and 4 were significantly greater than the 0 week values. However, all mean values were within the stated normal ranges (Table 9). There were also a number of significant differences in blood chemistries mean values as can be seen in Table 10. The variables with significant differences ($p < 0.05$) were Na, K, Cl, Ca, HDL-Cho, Albumin and HbA1c; however, again the mean values were within the normal range. No differences were observed in any urinalysis values (Table 11). At all sampling times during the study, including week 0 and 2 weeks after the cessation of consumption of IMD, there were individual subjects that had one or a few values that were outside the normal range. Review of these subjects, times, variables and values showed no consistent pattern. If the initial values were either high or low at week 0, these values continued to be so throughout the study, with only slight changes. The investigator concluded that none of these differences were clinically relevant because the values were considered to be within the range of normal daily life. The timing appeared random, and the mean values were within the normal range. One subject reported mild hard stools from the day after the start of IMD intake, but this condition disappeared during the course of the study, and the investigator considered it as not significant to the subject’s health. The subjects reported no other adverse effects.

Twelve-week low-dose ingestion study in humans

The results of physiological tests, hematological tests, blood chemistry tests, and urine analysis are shown in Tables 12, 13, 14, and 15, respectively. The intake compliance rate of IMD was 98.7 ± 2.7%. Significant differences ($p < 0.05$) in physical measurements in comparison to week 0 included a decrease in the diastolic blood pressure at week 4 and 8, while the mean pulse rate was significantly increased at week 4 (Table 12). These changes were considered acceptable by the investigator since the

Table 8. Physical data from the four-week high-dose IMD ingestion study in humans.

	n	0 week	2 weeks	4 weeks	2 weeks after
Body weight (kg)	20	60.78 ± 7.58	61.33 ± 7.47*	61.33 ± 7.71	61.45 ± 7.45*
BMI (kg/m^2)	20	22.69 ± 1.84	22.90 ± 1.88*	22.89 ± 1.93	22.95 ± 1.88*
SBP (mmHg)	20	117.9 ± 12.1	115.6 ± 13.0	117.1 ± 12.3	118.9 ± 12.9
DBP (mmHg)	20	68.2 ± 9.9	64.7 ± 12.1	69.0 ± 10.3	67.3 ± 12.5
Pulse (/min)	20	77.5 ± 9.6	75.7 ± 8.2	76.6 ± 8.3	76.7 ± 11.3

Values are mean ± S.D. *Significantly different from 0 week using paired t-test with Bonferroni correction at $p < 0.05$.

Table 9. Hematologic data from the four-week high-dose IMD ingestion study in humans.

	Reference values		Sex	n	0 week	2 weeks	4 weeks	2 weeks after
WBC (/μL)		3300-9000		20	5775.0 ± 1186.7	5275.0 ± 1129.5*	5240.0 ± 928.7	5370.0 ± 1223.1
RBC (×10^4/μL)	Male	430-570	Male	9	476.4 ± 40.7	473.8 ± 40.3	477.0 ± 43.9	487.8 ± 40.8
	Female	380-500	Female	11	432.1 ± 21.1	426.2 ± 21.8	435.7 ± 20.9	442.2 ± 23.5*
			All	20	452.1 ± 38.0	447.6 ± 39.0	454.3 ± 38.5	462.7 ± 39.1*
HGB (g/dL)	Male	13.5-17.5	Male	9	14.97 ± 1.13	14.96 ± 0.89	15.11 ± 1.07	15.22 ± 1.09
	Female	11.5-15.0	Female	11	12.83 ± 0.91	12.88 ± 0.88	13.12 ± 1.10	13.10 ± 0.90*
			All	20	13.79 ± 1.47	13.82 ± 1.36	14.02 ± 1.47	14.06 ± 1.45*
HCT (%)	Male	39.7-52.4	Male	9	44.96 ± 2.77	44.27 ± 2.23	44.86 ± 2.23	45.42 ± 1.96
	Female	34.8-45.0	Female	11	40.25 ± 2.50	39.32 ± 2.35*	40.46 ± 2.90	40.52 ± 2.52
			All	20	42.37 ± 3.50	41.55 ± 3.37*	42.44 ± 3.40	42.73 ± 3.35
MCV (fL)		85-102		20	93.8 ± 4.3	93.0 ± 4.5	93.7 ± 5.2	92.5 ± 5.1*
MCH (pg)		28.0-34.0		20	30.49 ± 1.85	30.89 ± 1.93*	30.84 ± 1.90*	30.38 ± 1.92
MCHC (%)		30.2-35.1		20	32.49 ± 0.95	33.21 ± 0.91*	32.97 ± 1.00*	32.85 ± 1.21
PLT (×10^4/μL)		14.0-34.0		20	24.82 ± 3.89	25.60 ± 4.17	25.78 ± 5.15	25.63 ± 4.79

Values are mean ± S.D. *Significantly different from 0 week using paired t-test with Bonferroni correction at $p < 0.05$.

changes were within the range of normal daily life activities. There were also significant differences ($p < 0.05$) in a number of hematologic, blood chemistry and urological variables. Tables 13, 14 and 15 provide the values at the specific sampling times and by gender groups, if applicable. The following is a list of the variables in which significant differences were observed from the week 0 mean values. The MCV and MCHC at week 4; the MCV, MCHC, Cl, serum amylase, HbA1c, and urine creatinine at week 8; the HCT (male subjects), MCV, MCHC, TG, Na, and glucose at week 12; and in the HGB (all subjects), MCV, MCH, MCHC, PLT, Cl, iP, serum amylase, glucose, and HbA1c at 4 weeks after stopping consumption, as compared with week 0 values. All mean values that were significantly different were within the reference ranges for each variable. At all sampling times during the study, including week 0 and 4 weeks after the cessation of consumption of IMD, there were individual subjects that had one or a few values that were outside the normal range. Review of these subjects, times, variables and values showed no consistent pattern. If the initial values were either high or low at week 0, these values continued to be so throughout the study, with only slight changes. The investigator concluded that none of these differences were clinically relevant because the values were considered to be within the range of normal daily life. The timing appeared random, and the mean values were within the normal range. No adverse events were reported during the observation period (week 0 to 4 weeks after stopping IMD consumption). The investigator concluded that IMD had no adverse effects on the subjects' general health.

DISCUSSION

As part of the safety evaluation of IMD, the following tests and studies were conducted: A bacterial reverse mutation test, a micronucleus test, a chromosome aberration test, an acute oral toxicity study, a 90-day repeated-oral dose toxicity study, an evaluation study on the no observed effect level for loose stools in humans, a 4-week high-dose ingestion study in humans, and a 12-week low-dose ingestion study in humans.

Review of the bacterial reverse, micronucleus and chromosomal aberration tests indicated no effect at any dose and under any conditions of use. The negative and positive controls demonstrated that the assays performed as designed. These results demonstrated the under the standard conditions of these assays that IMD is not gen-

Table 10. Blood chemistry data from the four-week high-dose IMD ingestion study in humans.

	Reference values		Sex	n	0 week	2 weeks	4 weeks	2 weeks after
T-Bilirubin (mg/dL)		0.2-1.2		20	0.86 ± 0.32	0.81 ± 0.27	0.82 ± 0.29	0.84 ± 0.28
AST (U/L)		10-40		20	18.9 ± 4.3	19.5 ± 5.0	18.9 ± 4.5	19.7 ± 5.7
ALT (U/L)		5-45		20	14.6 ± 7.0	16.2 ± 7.4	15.9 ± 7.4	15.3 ± 6.1
ALP (U/L)		100-325		20	192.7 ± 54.0	196.0 ± 50.4	192.6 ± 54.8	191.8 ± 54.0
LDH (U/L)		120-240		20	182.7 ± 28.7	182.5 ± 29.3	178.7 ± 25.0	176.8 ± 31.3
γ-GTP (U/L)	Male	≤80	Male	9	27.8 ± 16.7	30.8 ± 20.3	24.9 ± 17.8	27.1 ± 15.7
	Female	≤30	Female	11	16.5 ± 12.1	18.2 ± 11.1	18.0 ± 11.0	18.9 ± 16.5
			All	20	21.6 ± 15.1	23.9 ± 16.7	23.2 ± 15.2	22.6 ± 16.3
CPK (U/L)	Male	60-270	Male	9	107.3 ± 26.8	111.4 ± 38.0	114.8 ± 35.1	103.2 ± 29.1
	Female	40-150	Female	11	75.5 ± 33.5	81.1 ± 35.6	81.1 ± 25.6	84.6 ± 35.0
			All	20	89.9 ± 34.0	94.8 ± 38.9	96.3 ± 34.1	93.0 ± 33.0
T-Protein (g/dL)		6.7-8.3		20	7.34 ± 0.27	7.31 ± 0.26	7.23 ± 0.30	7.27 ± 0.32
Creatinine (mg/dL)	Male	0.61-1.04	Male	9	0.833 ± 0.140	0.847 ± 0.103	0.837 ± 0.127	0.842 ± 0.125
	Female	0.47-0.79	Female	11	0.681 ± 0.080	0.663 ± 0.092	0.682 ± 0.100	0.673 ± 0.073
			All	20	0.750 ± 0.133	0.746 ± 0.133	0.752 ± 0.135	0.749 ± 0.130
Urea nitrogen (mg/dL)		8.0-20.0		20	13.14 ± 3.08	12.16 ± 2.94	12.10 ± 3.30	11.51 ± 3.02
Uric acid (mg/dL)	Male	3.8-7.0	Male	9	5.64 ± 1.26	5.86 ± 1.34	5.76 ± 1.66	5.38 ± 1.29
	Female	2.5-7.0	Female	11	4.75 ± 0.78	4.80 ± 0.78	4.71 ± 0.82	4.74 ± 0.83
			All	20	5.16 ± 1.09	5.28 ± 1.17	5.18 ± 1.34	5.03 ± 1.08
T-Cho (mg/dL)		120-219		20	206.7 ± 30.9	201.6 ± 28.6	202.3 ± 35.8	206.0 ± 31.0
TG (mg/dL)		30-149		20	92.4 ± 39.1	82.7 ± 26.1	87.8 ± 28.4	83.8 ± 30.7
Na (mEq/L)		137-147		20	141.7 ± 1.6	140.3 ± 2.1*	140.6 ± 1.6*	140.5 ± 2.0*
K (mEq/L)		3.5-5.0		20	4.11 ± 0.22	4.19 ± 0.20	4.15 ± 0.27	4.24 ± 0.29*
Cl (mEq/L)		98-108		20	104.0 ± 1.8	103.0 ± 2.1*	103.6 ± 2.0	103.3 ± 2.2
Ca (mg/dL)		8.4-10.4		20	9.50 ± 0.31	9.50 ± 0.25	9.47 ± 0.33	9.33 ± 0.37*
iP (mg/dL)		2.5-4.5		20	3.49 ± 0.44	3.57 ± 0.40	3.67 ± 0.51	3.60 ± 0.37
Mg (mg/dL)		1.9-2.5		20	2.19 ± 0.15	2.18 ± 0.17	2.20 ± 0.16	2.16 ± 0.15
Fe (μg/dL)	Male	50-200	Male	9	138.4 ± 45.8	109.1 ± 30.5	112.1 ± 35.9	132.9 ± 53.5
	Female	40-180	Female	11	101.9 ± 34.1	105.5 ± 37.1	92.6 ± 43.4	98.3 ± 42.2
			All	20	118.4 ± 42.9	107.2 ± 33.5	101.4 ± 40.4	113.9 ± 49.6
Serum amylase (U/L)		40-122		20	79.4 ± 19.6	82.9 ± 19.0	80.9 ± 19.3	83.1 ± 18.8
HDL-Cho (mg/dL)		40-85	Male	9	65.3 ± 21.9	66.8 ± 23.6	62.9 ± 22.8	68.3 ± 23.2
		40-95	Female	11	71.5 ± 10.8	75.1 ± 11.2	71.8 ± 12.9	76.1 ± 10.2*
			All	20	68.7 ± 16.6	71.4 ± 17.8	67.8 ± 18.1	72.6 ± 17.2*
Albumin (g/dL)		3.8-5.2		20	4.56 ± 0.32	4.54 ± 0.25	4.43 ± 0.29*	4.61 ± 0.28
Glucose (mg/dL)		70-109		20	82.9 ± 8.8	81.9 ± 10.5	81.5 ± 8.1	84.0 ± 8.4
LDL-Cho (mg/dL)		65-139		20	112.6 ± 25.4	112.6 ± 23.9	114.4 ± 27.5	114.2 ± 25.4
HbA1c (%)		4.6-6.2		20	5.19 ± 0.37	5.19 ± 0.37	5.32 ± 0.37*	5.40 ± 0.39*

Values are mean ± S.D. *Significantly different from 0 week using paired t-test with Bonferroni correction at $p < 0.05$.

otoxic.

An acute oral toxicity gavage study was performed in 5 rats with a single dose of 2,000 mg/kg of IMD. No indication of morbidity or mortality was observed; therefore, the oral LD_{50} of IMD was considered to be more than 2,000 mg/kg in this study.

For the evaluation of the subchronic safety of IMD continuous doses of 0, 100, 300 or 1,000 mg/kg/day were administered for 90 days to Crl:CD(SD) rats. While there were significant differences between dosage groups for hematologic, blood chemistry, urinalysis, and histologic variables, these were judged to be not associated with a toxicologic response. The justification for this conclusion is that the significant differences were not dose dependent and/or outside the range of normal values for the experimental animals used. Based on the above findings, the NOAEL of IMD was specified as 1,000 mg/kg/day for both male and female rats under the conditions of this study, which was the highest dose. This would be 60 g/day consumed by a 60-kg human.

Table 11. Urinalysis data from the four-week high-dose IMD ingestion study in humans.

	Reference values	n	0 week	2 weeks	4 weeks	2 weeks after
Protein	(-)	20	0.1 ± 0.3	0.2 ± 0.4	0.2 ± 0.5	0.0 ± 0.0
Urinary sugar	(-)	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Urobilinogen	(±)	20	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Bilirubin	(-)	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Occult blood	(-)	20	0.2 ± 0.5	0.4 ± 0.9	0.6 ± 0.9	0.4 ± 1.1
Ketone bodies	(-)	20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Specific gravity	1.006-1.030	20	1.0180 ± 0.0078	1.0141 ± 0.0097	1.0150 ± 0.0104	1.0145 ± 0.0083
pH	5.0-7.5	20	6.03 ± 0.53	6.28 ± 0.60	6.25 ± 0.53	6.20 ± 0.71
Creatinine (mg/dL)		20	131.520 ± 79.307	110.552 ± 96.191	111.687 ± 94.312	103.344 ± 77.167

Values are mean ± S.D. Protein, urinary sugar, urobilinogen, bilirubin, occult blood and ketone bodies were determined by urine test strip, and were refered to the scores (-: 0, ±: 1, +: 2, ++: 3, +++: 4). Protein: -, Negative. Urinary sugar: -, Negative. Urobilinogen: ±, Normal. Bilirubin : -, Negative. Occult blood: -, Negative. Ketone bodies: -, Negative.

Table 12. Physical data from the twelve-week low-dose IMD ingestion study in humans.

	n	0 week	4 weeks	8 weeks	12 weeks	4 weeks after
Body weight (kg)	19	62.33 ± 8.69	62.22 ± 8.61	62.53 ± 8.89	62.54 ± 8.79	62.65 ± 8.90
BMI (kg/m²)	19	23.37 ± 1.93	23.33 ± 1.90	23.44 ± 1.94	23.45 ± 1.87	23.48 ± 1.82
SBP (mmHg)	19	118.7 ± 6.8	117.0 ± 14.0	118.3 ± 11.4	118.7 ± 13.9	121.3 ± 13.4
DBP (mmHg)	19	73.9 ± 7.3	69.3 ± 10.9*	67.4 ± 12.5*	70.4 ± 12.7	71.2 ± 14.3
Pulses (/min)	19	70.4 ± 9.1	75.2 ± 8.9*	72.5 ± 6.1	73.1 ± 10.1	72.6 ± 8.3

Values are mean ± S.D. *Significantly different from 0 week using paired t-test with Bonferroni correction at $p < 0.05$.

Table 13. Hematologic data from the twelve-week low-dose IMD ingestion study in humans.

	Reference values		Sex	n	0 week	4 weeks	8 weeks	12 weeks	4 weeks after
WBC (/μL)		3300-9000		19	5736.8 ± 1298.0	5721.1 ± 1451.3	5505.3 ± 1451.6	6178.9 ± 1639.6	5794.7 ± 1443.9
RBC (×10⁴/μL)	Male	430-570	Male	7	475.1 ± 29.0	486.0 ± 28.3	475.6 ± 26.1	473.9 ± 29.9	483.1 ± 29.4
	Female	380-500	Female	12	437.0 ± 25.8	440.8 ± 31.7	437.4 ± 28.5	441.1 ± 29.5	443.3 ± 24.0
			All	19	451.1 ± 32.3	457.5 ± 37.2	451.5 ± 32.9	453.2 ± 33.1	457.9 ± 32.1
HGB (g/dL)	Male	13.5-17.5	Male	7	14.40 ± 1.07	14.84 ± 1.18	14.40 ± 0.97	14.41 ± 1.06	14.84 ± 1.14
	Female	11.5-15.0	Female	12	12.76 ± 0.63	12.96 ± 0.62	12.89 ± 0.69	13.00 ± 0.70	13.13 ± 0.79
			All	19	13.36 ± 1.13	13.65 ± 1.25	13.45 ± 1.08	13.52 ± 1.08	13.76 ± 1.24*
HCT (%)	Male	39.7-52.4	Male	7	44.13 ± 3.08	44.60 ± 2.96	43.80 ± 3.03	43.44 ± 3.11*	44.49 ± 2.81
	Female	34.8-45.0	Female	12	40.24 ± 1.59	40.01 ± 2.21	39.86 ± 2.11	39.87 ± 2.16	40.23 ± 2.31
			All	19	41.67 ± 2.90	41.70 ± 3.33	41.31 ± 3.10	41.18 ± 3.04	41.80 ± 3.22
MCV (fL)		85-102		19	92.5 ± 3.5	91.2 ± 3.7*	91.5 ± 3.5*	90.9 ± 3.5*	91.3 ± 3.1*
MCH (pg)		28.0-34.0		19	29.64 ± 1.39	29.85 ± 1.40	29.81 ± 1.42	29.86 ± 1.37	30.04 ± 1.38*
MCHC (%)		30.2-35.1		19	32.05 ± 0.88	32.73 ± 0.88*	32.55 ± 0.69*	32.83 ± 0.69*	32.91 ± 0.76*
PLT (×10⁴/μL)		14.0-34.0		19	26.49 ± 4.03	27.06 ± 4.27	26.42 ± 4.56	26.29 ± 4.26	27.78 ± 3.87*

Values are mean ± S.D. *Significantly different from 0 week using paired t-test with Bonferroni correction at $p < 0.05$.

In an initial gastrointestinal tolerance study a NOAEL for loose stools could not be calculated by the method of Oku *et al* because only one subject reported "loose stools", meaning muddy or watery, after consuming the highest dose (70 g). In a second study no subjects developed loose stools at a dose of 0.8 g/kg-BW, but loose stools were randomly observed in 4 subjects out of 19 at doses of 1.0 g/kg-BW or higher. Thus, the NOAEL of IMD for loose stools was estimated to be 0.8 g/kg-BW. The occasionally observed abdominal symptoms were all mild. The estimated NOAEL for loose stools (0.8 g/kg-BW) corresponds to 48 g for a 60-kg person. It is reported that the average daily intake of dietary fiber is 14.7 g in a Japanese population aged 20 years or older (Ministry of Health, Labour and Welfare, 2013), and 15.9 g in Americans sampled between 2007 and 2008 (King *et al.*, 2012). It is unlikely that human adults would consume 48 grams of IMD dietary fiber, which is approximately 3 times

Table 14. Blood chemistry data from the twelve-week low-dose IMD ingestion study in humans.

	Reference values		Sex	n	0 week	4 weeks	8 weeks	12 weeks	4 weeks after
T-Bilirubin (mg/dL)		0.2-1.2		19	0.70 ± 0.24	0.70 ± 0.15	0.73 ± 0.22	0.69 ± 0.18	0.64 ± 0.22
AST (U/L)		10-40		19	18.3 ± 3.4	19.6 ± 4.9	19.4 ± 5.2	20.9 ± 9.1	20.6 ± 7.9
ALT (U/L)		5-45		19	15.8 ± 6.7	18.7 ± 10.5	18.2 ± 10.5	20.4 ± 17.0	20.1 ± 12.5
ALP (U/L)		100-325		19	170.8 ± 35.7	182.7 ± 47.5	193.3 ± 64.4	178.9 ± 42.5	181.4 ± 36.5
LDH (U/L)		120-240		19	178.2 ± 24.9	175.3 ± 21.3	173.0 ± 21.6	170.8 ± 23.4	176.1 ± 21.6
γ-GTP (U/L)	Male	≤80	Male	7	26.4 ± 19.6	29.1 ± 20.8	29.3 ± 22.1	30.1 ± 22.0	27.7 ± 18.3
	Female	≤30	Female	12	15.3 ± 4.6	18.8 ± 9.4	18.2 ± 9.8	19.0 ± 14.7	17.6 ± 6.4
			All	19	19.4 ± 13.1	22.6 ± 15.0	22.3 ± 15.9	23.1 ± 18.0	21.3 ± 12.7
CPK (U/L)	Male	60-270	Male	7	136.3 ± 46.2	110.7 ± 13.2	108.4 ± 12.6	111.6 ± 18.1	113.9 ± 18.8
	Female	40-150	Female	12	75.6 ± 33.0	86.6 ± 51.7	117.6 ± 124.4	87.8 ± 43.8	79.4 ± 33.4
			All	19	97.9 ± 47.8	95.5 ± 42.8	114.2 ± 97.6	96.6 ± 37.7	92.1 ± 33.0
T-Protein (g/dL)		6.7-8.3		19	7.16 ± 0.34	7.23 ± 0.26	7.24 ± 0.34	7.27 ± 0.36	7.25 ± 0.31
Creatinine (mg/dL)	Male	0.61-1.04	Male	7	0.844 ± 0.129	0.824 ± 0.098	0.840 ± 0.117	0.841 ± 0.092	0.796 ± 0.086
	Female	0.47-0.79	Female	12	0.578 ± 0.072	0.568 ± 0.087	0.595 ± 0.075	0.598 ± 0.079	0.589 ± 0.068
			All	19	0.676 ± 0.162	0.662 ± 0.155	0.685 ± 0.151	0.687 ± 0.146	0.665 ± 0.125
Urea nitrogen (mg/dL)		8.0-20.0		19	11.72 ± 2.69	11.43 ± 2.75	11.68 ± 2.09	11.49 ± 3.10	12.08 ± 3.49
Uric acid (mg/dL)	Male	3.8-7.0	Male	7	5.99 ± 0.78	6.11 ± 1.03	5.93 ± 1.13	6.06 ± 0.71	5.73 ± 0.97
	Female	2.5-7.0	Female	12	4.78 ± 0.93	4.63 ± 0.84	4.78 ± 0.80	4.69 ± 0.92	4.66 ± 1.04
			All	19	5.23 ± 1.04	5.17 ± 1.15	5.21 ± 1.07	5.19 ± 1.07	5.05 ± 1.12
T-Cho (mg/dL)		120-219		19	195.6 ± 28.9	202.1 ± 28.9	202.0 ± 29.5	204.9 ± 36.3	198.9 ± 26.5
TG (mg/dL)		30-149		19	87.5 ± 32.0	94.1 ± 39.1	84.1 ± 43.7	67.3 ± 25.1*	75.3 ± 32.5
Na (mEq/L)		137-147		19	140.8 ± 1.5	141.3 ± 1.3	140.2 ± 1.5	139.5 ± 1.1*	139.9 ± 1.9
K (mEq/L)		3.5-5.0		19	4.08 ± 0.27	4.13 ± 0.15	4.22 ± 0.24	4.07 ± 0.27	4.27 ± 0.28
Cl (mEq/L)		98-108		19	104.6 ± 2.2	103.7 ± 2.0	103.1 ± 1.4*	103.7 ± 2.3	103.2 ± 2.3*
Ca (mg/dL)		8.4-10.4		19	9.22 ± 0.26	9.31 ± 0.33	9.32 ± 0.38	9.36 ± 0.38	9.34 ± 0.45
iP (mg/dL)		2.5-4.5		19	3.38 ± 0.30	3.44 ± 0.36	3.45 ± 0.32	3.47 ± 0.52	3.59 ± 0.37*
Mg (mg/dL)		1.9-2.5		19	2.19 ± 0.12	2.14 ± 0.11	2.19 ± 0.12	2.13 ± 0.13	2.18 ± 0.10
Fe (μg/dL)	Male	50-200	Male	7	100.1 ± 46.4	104.7 ± 36.1	93.1 ± 32.3	93.1 ± 20.2	81.7 ± 26.8
	Female	40-180	Female	12	87.4 ± 34.4	107.0 ± 41.7	103.4 ± 36.4	104.5 ± 43.1	95.7 ± 52.5
			All	19	92.1 ± 38.5	106.2 ± 38.7	99.6 ± 34.4	100.3 ± 36.1	90.5 ± 44.4
Serum amylase (U/L)		40-122		19	68.4 ± 18.2	73.8 ± 23.1	73.9 ± 20.3*	70.2 ± 22.3	72.6 ± 18.9*
HDL-Cho (mg/dL)		40-85	Male	7	57.7 ± 14.9	55.0 ± 13.2	57.0 ± 14.6	59.1 ± 15.3	54.0 ± 13.1
		40-95	Female	12	64.0 ± 10.7	66.8 ± 12.4	68.3 ± 13.7	69.3 ± 13.6	66.8 ± 12.6
			All	19	61.7 ± 12.4	62.5 ± 13.6	64.2 ± 14.8	65.6 ± 14.7	62.1 ± 14.0
Albumin (g/dL)		3.8-5.2		19	4.38 ± 0.30	4.29 ± 0.24	4.44 ± 0.26	4.45 ± 0.30	4.41 ± 0.23
Glucose (mg/dL)		70-109		19	79.3 ± 5.8	82.7 ± 9.1	81.2 ± 7.0	82.2 ± 5.8*	83.2 ± 5.4*
LDL-Cho (mg/dL)		65-139		19	114.1 ± 24.4	119.7 ± 22.1	118.0 ± 25.3	124.3 ± 29.6	117.5 ± 21.6
HbA1c (%)		4.6-6.2		19	5.32 ± 0.24	5.35 ± 0.29	5.44 ± 0.33*	5.39 ± 0.32	5.41 ± 0.29*

Values are mean ± S.D. *Significantly different from 0 week using paired t-test with Bonferroni correction at $p < 0.05$.

the normal daily values, per day. Thus, the NOAEL of 0.8 g/kg-BW seems to be an acceptable level for usual intake. In general, an intake of a large amount of other indigestible carbohydrates at one time have been reported to cause loose stools (Yoshikawa *et al.*, 2013). The NOAELs for loose stools of various types of indigestible carbohydrates are reported as follows: *Sugar alcohols*—0.22 g/kg-BW for sorbitol (Yamazaki *et al.*, 2011) and 0.3 g/kg-BW for xylitol (Koizumi *et al.*, 1998); *Oligosaccharides*—0.3 g/kg-BW (male) and 0.4 g/kg-BW (female) for fructo-oligosaccharide (Hata and Nakajima, 1985), 0.30 g/kg-BW for galacto-oligosaccharide (Kimura *et al.*, 2004); *Soluble dietary fiber*—0.7 g/kg-BW for polydextrose (Flood *et al.*, 2004), and 1.0 g/kg-BW (male) and more than 1.1 g/kg-BW (female) for resistant maltodextrin (Kishimoto *et al.*, 2013). Comparing these common indigestible carbohydrates with IMD, the tolerance of IMD appears to be considerably higher than sugar alcohols and oligosaccharides, and essentially the same as soluble dietary fiber. Further, IMD consumption resulted in

Table 15. Urinalysis data from the twelve-week low-dose IMD ingestion study in humans.

	Reference values	n	0 week	4 weeks	8 weeks	12 weeks	4 weeks after
Protein	(-)	19	0.2 ± 0.4	0.2 ± 0.4	0.0 ± 0.0	0.3 ± 0.7	0.0 ± 0.0
Urinary sugar	(-)	19	0.1 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Urobilinogen	(±)	19	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Bilirubin	(-)	19	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Occult blood	(-)	19	0.3 ± 1.0	0.2 ± 0.5	0.1 ± 0.2	0.3 ± 0.9	0.3 ± 0.8
Ketone bodies	(-)	19	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.5
Specific gravity	1.006-1.030	19	1.0224 ± 0.0078	1.0207 ± 0.0091	1.0163 ± 0.0081	1.0187 ± 0.0088	1.0190 ± 0.0093
pH	5.0-7.5	19	6.24 ± 0.54	6.16 ± 0.69	6.37 ± 0.72	6.39 ± 0.59	6.24 ± 0.65
Creatinine (mg/dL)		19	185.327 ± 79.011	156.127 ± 73.859	108.843 ± 59.973*	137.313 ± 78.450	129.951 ± 95.500

Values are mean ± S.D. *Significantly different from 0 week using paired t-test with Bonferroni correction at $p < 0.05$. Protein, urinary sugar, urobilinogen, bilirubin, occult blood and ketone bodies were determined by urine test strip, and were refered to the scores (-: 0, ±: 1, +: 2, ++: 3, +++: 4). Protein: -, Negative. Urinary sugar: -, Negative. Urobilinogen: ±, Normal. Bilirubin : -, Negative. Occult blood: -, Negative. Ketone bodies: -, Negative.

loose stools in 10% of the subjects at up to 1.8 g/kg-BW and only 20% at up to 2.4 g/kg-BW. Therefore even at a dose of 144 grams in a 60-kg person only 20% had loose stools. Based on the above, IMD is considered to have little, if any, negative effects on the digestive system.

In the 4-week high-dose (30 g/day) ingestion study in humans, there were significant changes in several variables after 4 weeks of consumption when compared to the week 0 values. Examination of these hematologic and blood chemistries variables showed that that changes were relatively small, and the mean values were within the normal range. Some subjects had a few values outside the normal range at various sampling time, including week 0; however, these laboratory values remained relatively consistent throughout the study. After review by the study supervisors, the various changes were considered clinically insignificant.

In the 12-week low-dose (10 g/day) ingestion study in humans there were a number of values that had significant mean changes from week 0. Examination of the laboratory variables showed that these were relatively small, and were still within the various standard range values. As with the previous 4-week study there were some subjects with values outside the normal range, including week 0. These subjects had little changes in these values. The observed changes were considered clinically insignificant. There were no adverse events reported during the observation period, and the investigator considered that IMD had no adverse effects on the subjects' general health. Therefore, IMD was also considered safe in this study.

Considering its physical structure containing only D-glucose units bound by alpha linkages, IMD appears substantially equivalent to other common soluble dietary fibers with known safety profiles. These include resistant dextrin, resistant maltodextrin, polydextrose and pullulan (Wils *et al.*, 2008; Wakabayashi *et al.*, 1992; Flood *et al.*, 2004; Kimoto *et al.*, 1997).

The glycosidic bonds in commonly used starches, dextrins and maltodextrins, are almost exclusively α-1,4 and α-1,6 linkages. These are usually digestible in the gastrointestinal tract. IMD consists of these bonds and also a small portion of α-1,3 bonds. The unique structural combination of these linkages are responsible for the non-digestible nature of IMD. While these bonds are found in resistant dextrin, resistant maltodextrin and polydextrose, they are not primarily thought to be the reason for their resistance to digestion (Tsusaki *et al.,* 2012). The resistance to digestion by resistant dextrin, resistant maltodextrin and polydextrose is thought to be the result of the various beta bonds in these molecules. IMD does not contain any beta bonds. These beta linkages are intrinsically non-digestible. Both alpha and beta bonds are found commonly in nature and are consumed as a large portion of the human diet. Pullulan is interesting because it is formed from only α-1,4 and α-1,6 linkages, and while the other resistant polyglucoses are branched like starch, it is a straight chain molecule with a molecular weight that is usually many times that of these other substances (Catley *et al.*, 1986). Dextrin and maltodextrin are formed by pyrolytic and/or enzymatic hydrolysis of starch where the manufacturing conditions can result in a wide range of product characteristics including the final product being either highly digestible or resistant to digestion (Okuma and Matsuda, 2002). The production process of polydextrose is via a condensation of set concentrations of D-glucose, sorbitol and citric acid. This results in a branched resistant polyglucose, consisting of a relatively high proportion of 1-6 linkages, many being beta (Flood *el al.*, 2004). The reducing ends of the polyglucose molecules

consist primarily of a molecule of sorbitol. Pullulan is typically fermented from starch syrup by the organism *Aureobasidium pullulans*. It consists of only glucose, and can be produced in a wide range of molecular weights depending on the culture conditions (Cately, 1971; Catley *et al., 1*986). While various manufacturing methods are employed to produce these polyglucose substances that are resistant to digestion (dietary fibers), all have substantially equivalent structures, and all have been reviewed by the FDA either in an FDA GRAS affirmation regulation, or are the subject of GRAS Notices for which “No questions” letters were provided.

Recently the FDA reviewed a GRAS Notice to consider the determination by the manufacturer, Hayashibara Co., Ltd., that IMD is GRAS by scientific procedures (proposed 21 CFR 170.36). The GRAS Notice included published safety data on the other resistant polyglucose substances mentioned in this article and references to their GRAS status (Wils *et al.*, 2008; Wakabayashi *et al.*, 1992; Flood *et al.*, 2004; Kimoto *et al.*, 1997). The GRAS Notice also included unpublished safety data on IMD as supporting data. In the Agency Response Letter it stated that, “the agency has no questions at this time regarding Hayashibara’s conclusion that isomaltodextrin is GRAS under the intended conditions of use.”

From the data presented in this paper, IMD has been demonstrated to have no toxicity and is well tolerated in humans. It is therefore concluded that IMD can be considered as generally recognized as safe for its intended use as a food ingredient when used in accordance with current Good Manufacturing Practices.

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Conflict of interest---- Two evaluation studies of NOAEL for loose stools was performed using employees of Hayashibara Co., Ltd.

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