Homeostasis as Regulated by Activated Macrophage. I. Lipopolysaccharide (LPS) from Wheat Flour: Isolation, Purification and Some Biological Activities

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Based on our new concept of ontogenic inflammation, we have sought a substance which can prime macrophage in terms of the endogenous production of tumor necrosis factor (TNF). A lipopolysaccharide (LPSw) was found in wheat flour, purified and characterized. The molecular size of LPSw was about 5 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and it contained 3-deoxy-D-manno-octulosonic acid: 1, hexosamine: 4 and one phosphorus in a single molecule. LPSw can prime macrophage to release TNF when given intradermally, percutaneously or even orally in mice as well as in humans, in exactly the same way as intravenous administration of interferon γ.

Keywords tumor necrosis factor; lipopolysaccharide; macrophage; flour; homeostasis

Introduction

The original concept of Metchnikoff concerning inflammation indicates that inflammation is initiated by macrophages and that an activated macrophage is persistently observed in inflammation phylogenically throughout the animal kingdom. We have developed this concept further: inflammation may also take place ontogenically. And we have succeeded in obtaining a state which resembles conventional inflammation in adults induced at emergency, as indicated by the endogeneous production of tumor necrosis factor (TNF), through the fetal period of mouse ontogenesis.[1] We call this "ontogenic inflammation".

Throughout the fetal period, cells of all tissues remain at the primed stage, ready to produce TNF on triggering without any foreign stimulant, which shows that ontogenic inflammation, especially in this primed stage of all tissue cells, probably regulates the homeostasis of embryogenesis. Thus, this must be an essential prerequisite to the regulation of ontogenesis.

If the above mentioned ontogenic inflammation can be appropriately reproduced in adults, especially in patients with intractable diseases, it might be possible to restore homeostasis from a distorted or deviated state. In adults, the cells to be primed are restricted to macrophages and their analogous cells, and once macrophages are adequately activated we can expect them to do the work of restoring homeostasis.

We have been involved in extensive studies of the endogenous production of TNF in adults.[2-8] Free TNF can be induced endogeneously and is now being clinically applied in cancer therapy[9-11]; we call this exogenous/endogenous TNF (EET) therapy.

The next target is to reproduce the primed stage of TNF production in an adult. The most successful procedure so far in this treatment to achieve the primed stage of macrophage is employment of a reversible primer such as interferon γ (IFN-γ),[3,4] TNF-α,[5] or a small amount of lipopolysaccharide (LPS) (IFN-γ type in Fig. 1). However, all these primers must be administered parenterally and they also carry with them some potential side effects.

We began anew to seek a substance which could induce the primed stage without harm, giving particular attention to the following points: 1. a primer of the IFN-γ type as shown in Fig. 1 was sought, 2. screening was to be done on foodstuffs, especially on those of plant origin, and 3. as much as possible, selected samples were to be assayed orally, percutaneously or intradermally.

At an early period of this random screening, we discovered a new substance from wheat flour. The present report describes the identification of the sample as LPS, its purification and some of its biological activities. LPS derived from concomitant bacteria is also discussed.

Materials and Methods

Samples of Wheat Flour Various types of wheat flour and wheat were provided by the Chiba Flour Milling Co. (Chiba, Japan). These were: Dark Northern Springs (DNS) imported from the U.S.A., Hard Red Winter (HRW-SH, -HP, -O) from the U.S.A., Western White (WW) from the U.S.A., No. 1 Canadian Western Red Wheat (1CW) from Canada, Australian Standard White (ASW) from Australia, and Horoshiri (H) harvested in Japan. Water extracts of wheat flour (wheat flour: water = 1 : 2.6) and partially purified water extracts (this preparation, called LPSw-H, contained 0.01—0.1% of LPSw) were prepared by the Chiba Flour Milling Co.

Chemical Reagents Toxicolor (a kit preparation of the conventional Limulus test) and Endospecy (Limulus test-kit without the G-factor), which reacts specifically with LPS, were purchased from Seikagaku Co. (Tokyo, Japan). Almost all tests were done with Toxicolor, with a final check by Endospecy. ID test·EB-20 for identification of *Enterobacteriaceae*, standard agar, trypto-soya agar, triple sugar iron (TSI) agar, sulfide indole motility (SIM) agar, deoxyribonucleic acid (DNA) agar and cytochrome oxidase test strips were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Bacto tryptone, yeast extract, and LPS from *Escherichia coli* (*E. coli*) O127:B8 were purchased from Difco Lab. (Detroit, U.S.A.). LPS purified from *Salmonella minnesota* (*S. minnesota*) rough-mutants (R60, R345, R5, R7, and R595) were obtained from List Biological Laboratories Inc. (Campbell, U.S.A.). Pronase was from Kaken Pharmaceutical Co. (Tokyo, Japan), Q-Sepharose fast flow, sephadex-G25 fine, sepharose 6B, a protein low-molecular-weight electrophoresis kit, and a molecular weight marker (1860-101) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). rTNF-α (PAC 4D, 2×10^6 U/mg protein) was donated by Asahi Chemical Ind. (Tokyo, Japan). 3-Deoxy-D-manno-octulosonic acid (KDO) was from Sigma Chemical Co. (St. Louis, U.S.A.). Tricine used as a component of the running buffer of gel-electrophoresis was purchased from Research Organics Inc. (Cleveland, U.S.A.). OK-432 (Picibanil), an antitumor reagent prepared from *Streptococcus pyogenes*, was supplied by Chugai Pharmaceutical Co. (Tokyo, Japan), and the dosage unit was shown as KE; Klinische Einheit (1 KE = 0.1 mg). Other chemical reagents were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan).

Mice Male C3H/He mice 7 to 12 weeks of age were obtained from Shizuoka Experimental Animal Farm (Shizuoka, Japan), All mice were given a standard laboratory diet and water *ad libitum*.

Chemical Analysis of LPS The LPS samples were analyzed for their

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sugar content by the phenol–sulfuric acid method,[12] for hexosamine by the Elson-Morgan method,[13] for KDO by the diphenylamine method,[14] and for phosphorus by the method of Chen-Toribara.[15] Protein was analyzed by the Lowry method.[16] Nucleic acid was analyzed by absorbance at 260 nm.

Isolation of Bacteria from Wheat Flour and Identification of Isolates Water extracts were prepared from samples of various types of wheat flour. The extracts were inoculated on standard agar plates and incubated for 12 to 24 h at 30 °C. Apparently different colonies were randomly picked from agar plates, and single colonies were isolated. Bacteria isolated from each colony were tested for gram-negative (gram-staining), glucose-positive (TSI agar), and oxidase-negative (cytochrome oxidase test strip). Obtained gram-negative rod bacteria were further subjected to the following biochemical reactions using the ID test·EB-20 for identification of *Enterobacteriaceae*: indole production, Voges-Proskauer, H_2S production, esculin hydrolysis, activities of lysine decarboxylase, arginine dihydroxylase, ornithine decarboxylase, phenylalanine deaminase, β-galactosidase, urease, citrate and malonate utilization, acid production from inositol, sucrose, adonitol, raffinose, arabinose, mannitol, rhamnose, and sorbitol. Isolates were also inoculated into TSI agar, SIM agar and DNA agar to test the gas from glucose, motility, and deoxyribonuclease activity.

Standard bacteria were obtained from the Japan Collection of Microorganisms (The Institute of Physical and Chemical Research, Saitama, Japan). Bacteria used as standard were *Enterobacter agglomerans* (JCM 1236^T = ATCC 27155, ^{T} = type strain), *Enterobacter cloacae* (JCM 1232^T = ATCC 13047) and *Rahnella aquatilis* (JCM 1683^T = ATCC 33071). *E. agglomerans* has recently been ascribed to a new genus, *Pantoea agglomerans*.[17]

Bacterial Culture and LPS Purification Bacteria were grown for 12 to 18 h at 37 °C in a Luria culture broth containing per liter of medium (pH 7.0): 10 g of bacto tryptone, 5 g of yeast extract and 10 g of NaCl. Cultures were harvested by centrifugation at 3000 × ***g*** at 4 °C and washed once with distilled water. The cells were frozen and stored at −20 °C until use. LPS was extracted by the hot phenol method of Westphal *et al.*[18] Cells were extracted twice with a 45% (w/w) aqueous phenol at 65 to 68 °C for 20 min. The collected aqueous phase was dialyzed against distilled water for 2 d (four changes) at 4 °C, and applied to a Q-sepharose anion-exchange column. After stepwise elution, a 400 mM NaCl fraction was collected and ultrafiltrated (mol. weight cut off 200 kDa, Advantec Toyo, OHP-150). The final sample was frozen and dried. Purity of LPS was >95% (w/w) (protein contamination was less than 2% (w/w); nucleic acid contamination was less than 4% (w/w)).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) of LPS SDS-PAGE was carried out in 1.0 mm-thick 20% acrylamide slab gel according to the method of Schägger and Jagow.[19] This system, using tricine instead of glycine in a running buffer, produced high resolution peptides and proteins of low molecular weight (1—20 kDa). We employed the system for LPS of small molecular size. LPS bands were visualized with a silver staining kit (Bio-Rad Lab., Richmond, U.S.A.). Size marker standards for molecular size were those from an electophoresis kit for protein with low molecular weight and for peptide as well. These markers were confirmed to be valid by the fact that they matched the standard of LPS of *Salmonella minnesota* R-type mutant (R595: 3 kDa, R7: 3.5 kDa, R5: 3.7 kDa, R345: 3.9 kDa, R60: 4.1 kDa). This is an original procedure which has not previously been employed for the analysis of LPS.

Counting of Total Number of Bacteria Dead and Alive in Samples by Fluorescent Microscopic Observation We developed a new method to define the total bacterial number dead and alive in any kind of sample. This method is based on staining nucleic acid in a bacterial cell with acridine orange. The specific fluorescence was observed with a fluorescent microscope (Nikon, Optipot-2). The number of the fluorescent count was calibrated with a standardized count of bacteria, dead or alive, gram positive or negative, by colony forming units (CFU) and absolute number under the microscope. Accuracy of this method was 70—90%. Samples were suspended in distilled water at the concentration of 100 mg/ml, spread on a clean microscopeslide (1 μl/cm^2), air dried and fixed by heat, stained with 1% (w/v) acridine orange for 30 min at room temperature, washed, again air dried and observed at 400× or 1000× by the fluorescent microscope. To meet the definition of bacteria, objects had to exhibit orange-colored fluorescence with a fine edge and a length of about 0.5 to 10 μm. Counting was done at ten randomly chosen areas per sample and the number of bacteria was calculated statistically from the average counts.

TNF Assay TNF activity was assayed with L929 mouse fibroblast in the presence of actinomycin D (1 μg/ml) by the method of Ruff and Gifford,[20] with minor modification involving the extrapolation assay.[21] TNF activity was determined in units as dilution factors of the sample leading to the 50% survival ratio of L929 cells by rTNF-α (PAC 4D, 2×10^6 U/mg) as an internal reference in each assay.

***In Vivo* Priming Activity** Samples were dissolved in saline and administered intravenously (i.v.) or intradermally (i.d.) to C3H/He or BALB/c mice. Three hours later, 1 KE of OK-432 as a trigger was intravenously administered, and 2 h thereafter, serum was obtained and tested for TNF activity.

Ulcer Protection Experimental gastric ulcers in mice were induced by 100% ethanol. One hour prior to the administration of ethanol, sample (1 μg/mouse as Limulus activity) or bacterial LPS (200 μg/mouse) was administered orally. The preventive effect of the samples was compared with the ulcer index. Details will be reported later.

Results

Sorting of a New Substance At an early period of random screening of the samples obtained from vegetables and cereals, we recognized an active substance from wheat flour. A water extract of wheat flour given intravenously showed priming activity of the typical IFN-γ type (Fig. 1). A change in priming activity with time after administration of the sample also showed a typical IFN-γ type response which involves LPS of *E. coli* (Fig. 2). As this curve was exactly coincident with that of *E. coli* LPS, we tested polymixin B precipitation, did the Limulus test (G-factor was disregarded), and identified it as a LPS, which we called LPSw.

Purification of LPSw Purification was done pursuing the Limulus test-active substance. A spray-dried water

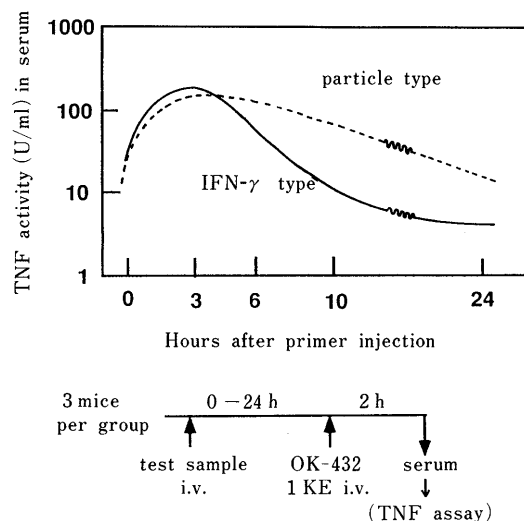

Fig. 1. Types of Primers for Endogenous TNF-Production

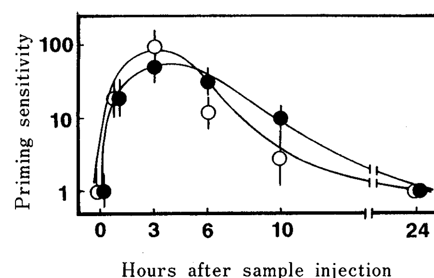

Fig. 2. Priming Effect of Samples Derived from Wheat Flour

●, water extract of wheat (1 ng of LPSw as Limulus activity/mouse); ○, *E. coli* LPS (1 ng/mouse). Symbols and bars represent the mean ± S.D., respectively. Priming sensitivity indicates relative priming activity of TNF induction to maximum induction. Experimental procedures are described in Materials and Methods.

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TABLE I. Purification Table of LPSw

Purification stage	Total LPS (mg)[a]	Total weight (mg)	Purification[b] factor (fold)	Yield (%)
Water extract of wheat flour	52	1000000	1	100
TCA treatment	9.5	1790	106	18
Gel filtration	6.0	300	400	12
Protease treatment	6.2	124	1000	12
Mono Q FPLC	3.5	3.5[c]	20000	6.7

a) Weight as expressed by Limulus activity. *b*) Purification factor was calculated from the following formula:

$$\text{purification factor} = \frac{\text{total LPS (mg) at each stage/total weight (mg) at each stage}}{\text{total LPS(mg) in water extract/total weight (mg) of water extract}}.$$

c) The purity of the final preparation of LPSw was estimated to be >91% (w/w), protein contamination was 8% of the dry weight in final preparation, and the nucleic acid contamination was less than 1% of the dry weight in final preparation.

TABLE II. Chemical Composition of LPSw Compared with That of Bacteria

Constituent	Content[a]		
	LPSw	*E. coli* LPS	Bacterium isolated from wheat flour
Phosphorus[b]	0.5	2	2
KDO[c]	0.7	0.8	2
Hexosamine[d]	3.5	5	2

a) Molecular weight of these samples is assumed to be 5 kDa. Each composition is expressed based on the molar ratio per 5 kDa. *b*) Chen–Toribara method.[15] *c*) 3-Deoxy-D-manno-octulosonic acid: diphenylamine method.[14] *d*) Elson–Morgan method.[13]

extract of wheat flour was suspended in water, extracted with 3% (w/v) trichloroacetic acid (TCA), resuspended in water, neutralized with NaOH and centrifuged. The procedure was repeated twice. The supernatant was concentrated with ultrafiltration (20 kDa, Advantec Toyo Co., Japan), chromatographed (sepharose 6B), treated with protease (pronase E) and ion-exchange-chromatographed (mono Q) with fast protein liquid chromatography (FPLC). The purification process and yield is shown in Table I.

All through the procedure, a Limulus positive substance was pursued. As shown in Table I, 1 kg of the starting material gave 3.5 mg of LPSw of 91% purity. The chemical composition and molecular size of LPSw was compared with other LPS derived from bacteria (Table II, Fig. 5). As shown later, the different point is that LPSw has a smaller molecular size of 5 kDa as compared with that of *E. coli* LPS, and it also has about one phosphorus per 5 kDa molecule (Table II).

In subsequent work in this series, we used the above sample. If necessary, a preparation called LPSw-H containing 0.01—0.1% LPSw which was condensed by ultrafiltration of a water extract of wheat flour was also used. In both cases, the amount of LPS indicated was based on a Limulus test (G-factor omitted).

Activity of LPSw by an Intradermal or Oral Route We found and reported in 1968 that LPS could activate macrophage when given intradermally,[22] and we therefore tested the priming activity of LPSw intradermally. Intradermal administration showed exactly the same pattern

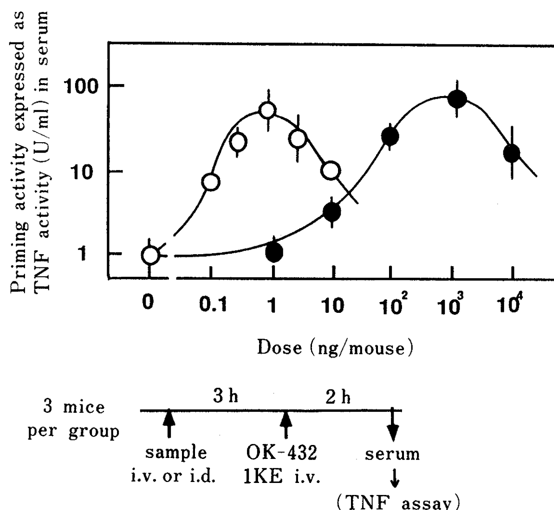

Fig. 3. Comparison of Priming Activity of LPSw by Administration Route

○, intravenous administration; ●, intradermal administration. Symbols represent the mean ± S.D., respectively.

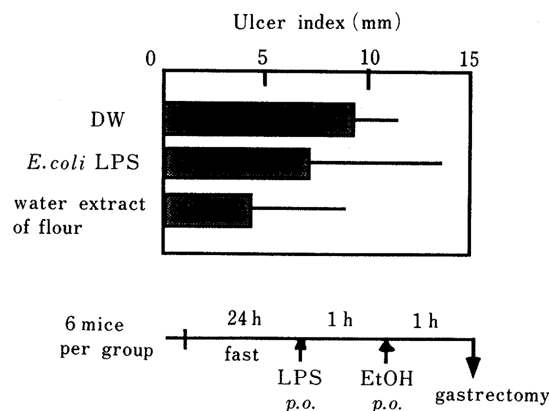

Fig. 4. Protective Effect of LPS on Ulcer Formation When Given Orally

Mice were fasted for 24 h, then 200 μl of samples (DW, 1 mg/ml of *E. coli* LPS, and 100 mg/ml of water extract of wheat flour which contained 50 μg/g of LPSw as Limulus activity) were administered, and after 1 h, 200 μl of 100% ethanol was administered orally. One hour later, gastrectomized and formalin fixed stomach samples were photographed and the length of the ulcer measured. The preventive effect of the samples were compared with the ulcer index. Each column represents the mean ± S.D., respectively.

as the intravenous route, though it required a dose 1000 times larger (Fig. 3). This suggested that percutaneous administration in a liniment might be available. When a liniment (50% glycerol solution) containing LPSw (1 μg/ml) was given percutaneously to humans, a marked increase of primed macrophage in the human body was observed. Details will be described elsewhere.

Protection of an ulcer induced by ethanol was tested using an oral administration of the water extract of wheat flour (LPSw-H). As shown in Fig. 4, LPSw-H, when given orally, protected ($p<0.1$) against ulcer-induction, though the efficacy was not as good as by the intradermal route. As will be shown in the following, an intradermal or percutaneous route is preferable, though the oral route is also possible. LPS of *E. coli* cannot be substituted.

The LD_{50} of LPSw as tested in BALB/c mice by the intravenous route is 3.2 mg/kg, the same value as in the case of *E. coli* LPS. Percutaneous testing did not allow estimation, althought we can at least say that it requires

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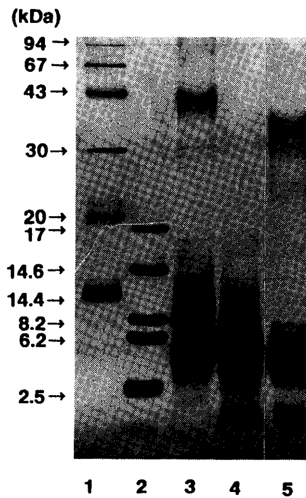

Fig. 5. Profile of SDS–PAGE of Purified Limulus-Positive Substance from Wheat Flour (LPSw) and LPS Purified from Gram-Negative Bacterium Isolated from Wheat Flour

Lane 1, 2: size marker, indicates molecular size (kDa); lane 3: 20 μg of *E. coli* LPS; lane 4: 20 μg of LPSw; lane 5: 20 μg of purified *P. agglomerans* LPS.

Table III. Gram-Negative Bacteria Isolated from Various Wheat Flours

Name of wheat flour	Place of origin	Bacteria isolates
Dark northern springs	U.S.A.	*Pantoea agglomerans* (70)[a]
Hard red winter-semi hard	U.S.A.	*Pantoea agglomerans* (60) *Enterobacter* sp.
Western white	U.S.A.	*Pantoea agglomerans* (60) *Serratia* sp.
No. 1 Canadian western red wheat	Canada	*Pantoea agglomerans* (70) *Enterobacter* sp. *Serratia* sp.
Australian standard white	Australia	*Pantoea agglomerans* (50)
Horoshiri	Japan	*Pantoea agglomerans* (40) *Serratia* sp.

a) Most abundant colony on nutrition agar plates. ( ): % of population/total isolates.

more than 100 mg per 4 cm^2. In the following works, the effective dose of LPSw in an adult human is shown as 1 μg/m^2. Therefore, the practical therapeutic index of LPSw (i.d.) in the human adult is more than 10^5. *E. coli* LPS cannot be substituted, since its molecular size is larger (Fig. 5).

Also, it is not effective by the oral route as will be described in another report of this series.

Bacteria Derived from Wheat Flour Contaminant bacteria in the preparations might have altered our results. We isolated the following 3 major bacterial colonies independently from wheat flour of different origins, derived from Canada, the U.S.A., Australia and Japan. Surprisingly, these same 3 species of gram-negative bacteria were definitely isolated, regardless of the production location. Identified by the ID-test (Table III), they were *Pantoea agglomerans*, *Enterobacter cloacae* and *Serratia ficaria*. In each case, the majority of colonies isolated were *Pantoea agglomerans* as determined by comparison with standard strains in the ID-test (Table IV). All these isolated strains of bacteria were grown in broth, and their LPS was purified by the conventional Westphal method.[18] Details will be given in a later paper.

Chemical compositions and the molecular weight of LPSw were compared with those of *Pantoea agglomerans* and of *E. coli*, and the behavior of LPSw was definitely different from those bacteria, both in chemical composition and gel pattern (Table II, Fig. 5).

The total number of bacteria dead and alive in wheat flour was counted by our fluorescent method and found to

Table IV. Phenotypic Characteristics of *Pantoea agglomerans*-like Strains[a]

Characteristic	*Bacterium* isolated from wheat flour	*Pantoea agglomerans* JCM 1236^T	*Enterobacter cloacae* JCM 1232^T	*Rahnella aquatilis* JCM 1683^T
Production of yellow pigment	+	+	−	−
Growth at 4 °C	−	−	−	+
at 37 °C	+	+	+	+
at 41 °C	−	−	−	−
Motility at 36 °C	−	−	+	+
Indole production	−	−	−	−
Voges–Proskauer reaction	+	+	+	+
H_2S production	−	−	−	−
Hydrolysis of esculin	+	+	+	+
Gas from D-glucose	−	−	+	+
Lysine decarboxylase	−	−	−	−
Arginine dihydrolase	−	−	+	−
Ornithine decarboxylase	−	−	+	−
Phenylalanine deaminase	−	−	−	−
Deoxyribonuclease	−	−	−	−
Oxidase (Kovacs')	−	−	−	−
β-Galactosidase	+	+	+	+
Urease	−	−	−	−
Utilization of:				
Citrate (Simmons)	+	+	+	+
Malonate	+	+	+	+
Acid production from:				
Inositol	−	−	−	−
Sucrose	+	+	+	+
Adonitol	−	−	−	−
Raffinose	−	−	+	+
L-Arabinose	+	+	+	−
D-Mannitol	+	+	+	+
L-Rhamnose	+	+	+	+
D-Sorbitol	−	−	+	+

a) +, reaction present in at least 90% of the strains within 24 to 48 h; −, reaction absent in at least 90% of the strains after 2 d. Tests were done at 30 °C unless otherwise indicated.

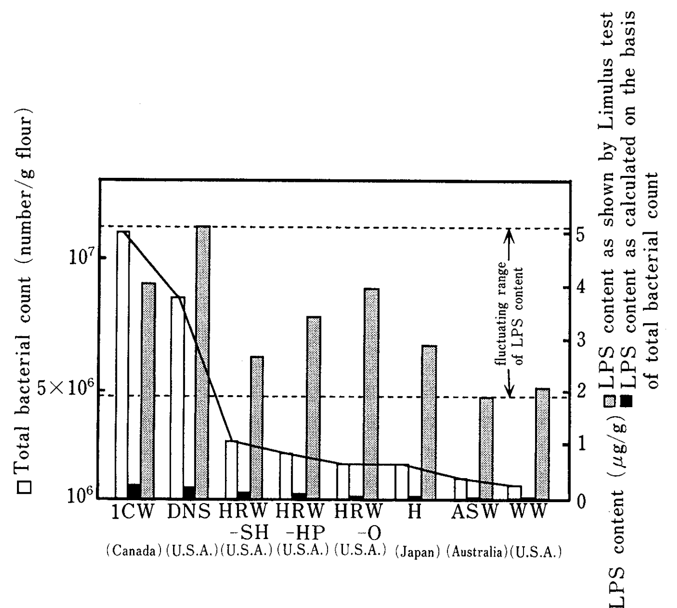

Fig. 6. LPS Content Calculated and Estimated Based on Total Number of Bacteria or Limulus Reaction in Wheat Flour

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be less than 10^7 cells/g in flour produced in 8 different places (Fig. 6). The number of concomitant bacteria differed. The open panel in Fig. 6 representing the bacterial count is shown in order of the amount. The amount of LPS from 1×10^7 bacterial cells is estimated as 0.3 μg at the maximal extraction. Based on this estimation, the amount of bacterial LPS contained per gram of flour from different locations is shown in Fig. 6 as a closed panel, according to the order of amount. Contrary to the order, the amount of LPSw per gram expressed by the hatched panels is far larger than the presumed amount of bacterial LPS (the maximal amount value was 0.3 μg), and the distribution fluctuates between 2 to 5 μg, irrespective of the total bacterial count in each flour. This confirms that LPS is definitely derived from the flour itself.

Discussion

Seeking a substance able to prime macrophages for endogenous production of TNF without harm, we identified a molecule from wheat flour as LPS. We call it LPSw, which has small molecular size, *ca.* 5 kDa, as compared with that of conventional *E. coli* LPS (10—30 kDa).

LPS can prime macrophages either by percutaneous or oral administration, and its practical therapeutic index by the non-parenteral route is more than 10^5 when LD_{50} and ED_{50} are compared. Based on our concept of ontogenic inflammation, LPSw has the capability to regulate the homeostasis of the human body and can possibly lead patients to recovery from intractable diseases. The priority of LPSw is that it is derived from wheat flour, a conventional non-toxic foodstuff, and that it can be used intradermally or percutaneously in a liniment without harm, and even be taken orally. The same cannot be said for *E. coli* LPS (Fig. 4).

The major associated bacteria in wheat flour cannot explain the existence of LPSw (Table II, Fig. 5): 1. bacterial LPS estimated from the total count of the presumed maximum of dead and living bacteria in the flour is less than 15% of the LPSw from wheat flour (Fig. 6), 2. chemical compositions and molecular weight of LPSw were definitely different from that of a bacterium (Table II, Fig. 5).

Each conventional drug known to date is taken for a particular disease, one drug for one disease. Contrary to this conventional concept, LPSw may be able to cure a variety of intractable diseases by its single function of priming the macrophages of a patient and thereby stimulating endogenous TNF production. This function will lead to regulation of the homeostasis of the patient. It is therefore considered that a single drug may have been found which can aid the cure of various intractable diseases, so long as it can activate macrophages appropriately. This seems to be an innovative drug concept, although ancient Chinese medicinal therapy may actually have been employing it for several thousands of years without understanding its functional mechanism. This feature will be discussed elsewhere.

We thus have established a new way to activate macrophages in adults up to the primed stage for TNF-production using LPSw by the non-parenteral route, orally or percutaneously. Since activated macrophages at the primed stage can appropriately regulate ontogenesis,[1] we believe that the homeostasis in adults can be restored from an inbalanced state in an intractable disease when LPSw is properly administered. As will be shown in following papers of this series, LPSw has been shown to be fairly effective in accomplishing this.

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