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Further Developments of the Therapy with Lipopolysaccharides of a Small Molecular Size on Various Intractable Diseases

A New Version of Coley's Toxin

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At the 3rd International Conference on TNF and Related Cytokines, 1990, held in Japan, Prof. Mizuno stated the significance of endogenous TNF production, especially that of the primed stage of macrophages for the maintenance of homeostasis based on the teleological role of endogenous TNF, which is an essential regulatory molecule during ontogenesis. We termed the regulatory mechanism driven by TNF 'ontogenic inflammation' [1]. Then, based on this concept of ontogenic inflammation we hypothesized that appropriate activation of macrophages can be effective in regulating homeostasis even in adults, and found some evidence that various intractable diseases can be restored by a single event, namely activation of macrophages using LPS of small molecular size [2–10].

Until now, we have tried to define characteristics of ontogenic inflammation at the molecular level intending to generalize the idea that ontogenic inflammation is a prototype of regulatory mechanism in adults to maintain homeostasis. Although ontogenic inflammation is one of several quite interesting concepts for the analysis of the biological role or clinical usefulness of endogenous TNF in adults, and also that some clues for the existence of TNF during late embryonal stage have been found [11], we have until now been unable to define what ontogenic inflammation is at a molecular basis and whether it would really exist throughout embryonal development or not.

Recently we found that ontogenic inflammation was actually provoked throughout embryonal development. In this report, ontogenic inflammation as a regulatory mechanism of embryonal development shall be specified first based on our new findings concerning ontogenic inflammation at the molecular level, and further insights into regulatory mechanisms in adults, such as the immune and neuroendocrine systems which are crucial for maintaining homeostasis in adults, will be demonstrated with recent data of the therapeutic usefulness of LPS with small molecular size.

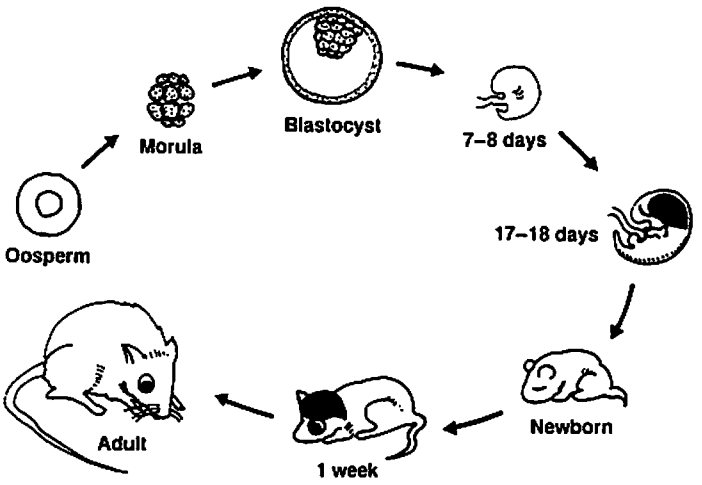


Fig. 1. Ontogenic inflammation in mice.

Ontogenic Inflammation at Molecular Level

Ontogenic inflammation, as introduced by us at the 3rd International Conference on TNF and Related Cytokine, is shown in figure 1. Ontogenic inflammation driven by TNF is apparent during mid-late embryonal development [11]. The question is whether the primed stage can be found during early embryonal development, i.e. before and at the time of implantation indicated by dots (fig. 1). If so, we can conclude that ontogenic inflammation is actually a regulatory mechanism and is provoked throughout embryonal development, and we can also assume that ontogenic inflammations shall be regarded as the prototype of regulatory mechanism for maintenance of homeostasis even in adults.

In order to examine characteristics of ontogenic inflammation at the molecular level, the findings of macrophages in the primed stage on a molecular basis offer a quite useful clue. In the primed stage, initially transcription of TNFα mRNA occurs and the precursor molecule of TNFα is actually expressed on cell membrane. Thus, the initial phenomenon of the primed stage can be characterized by the expression of TNF transcript in cells [12, 13]; details have been given by Tanabe et al. [14].

Based on the characteristics of the primed stage, we examined the expression of TNF and related cytokines during early embryonal development before and at the time of implantation. We tried to detect TNFα as

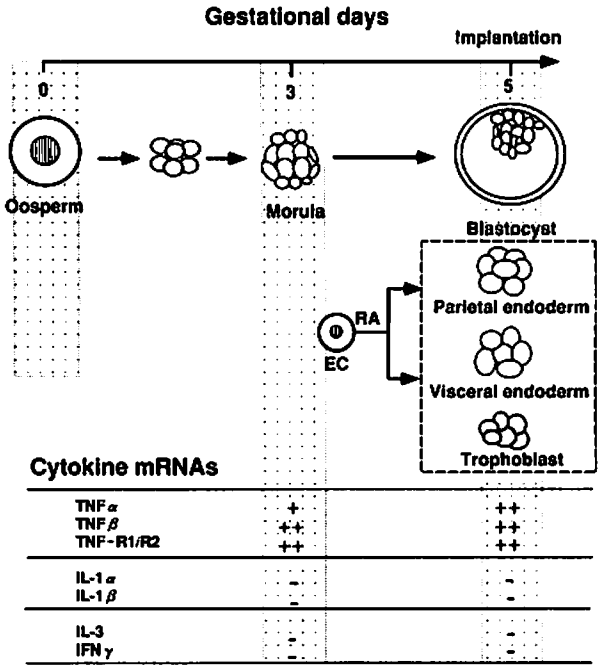


Fig. 2. Expression of cytokine mRNAs during early embryonal development.

well as TNFβ mRNA using the system of embryonal carcinoma (EC) cells and trophoblast cells.

As shown in the upper part of figure 2, normal embryonal development is in good accordance with the differentiation of EC cells and trophoblast cells. EC cells provide a good model to mimic early preimplantation embryos and the trophoblast cell is a model of early embryonal cells just before and at the time of implantation. If we could find the molecules which are commonly expressed during differentiation of EC cells and trophoblast cells, these molecules should play a regulatory role during embryogenesis.

As shown in the lower part of figure 2, both TNFα and TNFβ are expressed in all EC cells during differentiation and trophoblast cells examined with reverse transcripts/PCR method. On the other hand, monokines such as IL-1α and IL-1β, and lymphokines such as INF-γ and IL-3, which are known to form a cytokine network in the adult, are not expressed in early embryonal development [15]; details have been given by Kohchi et al. [16]. However, it should be stressed that TNFα and TNFβ are found as the first molecules to be expressed, at least after the morula period, and are

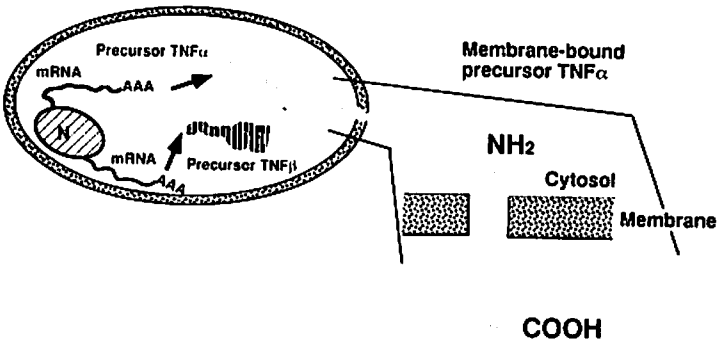


Fig. 3. Ontogenic inflammation at a molecular level.

continually expressed throughout embryonal development. The significant role of TNF administered exogenously for embryonal development before and after implantation is well known [17]. We found, however, for the first time, production of TNFα and TNFβ from the embryo itself throughout embryonal development. Two receptors for both TNFα and TNFβ are also expressed. These results demonstrated that a part of the signal transduction pathway by TNFα as well as TNFβ was established quite early in the embryonic stage. Also, since biological activity of TNFα was not observed in culture medium of EC cells and trophoblast cells, it may exist as a membrane-bound form. In other words, all cells are primed. TNFβ, known as the cytokine secreted from activated T lymphocytes, is considered to play a key role in inflammation as well as TNFα. But from our results the biological significance of TNFβ should be reconsidered in connection with its regulatory mechanisms even during embryonal development. Simultaneous expression of TNFα as well as TNFβ from all cells suggests that these two cytokines may act synergistically to regulate proper embryonal development. Based on our findings, ontogenic inflammation can be clearly distinguished from other inflammations in the adult by the expression of both TNFα and TNFβ mRNA in one cell. Taking all the results into consideration, we would like to propose the following definition of ontogenic inflammation on a molecular basis (fig. 3). First, characteristic of ontogenic inflammation is a simultaneous expression of TNFα and TNFβ mRNA in one cell. Second, TNFα should be expresed as a precursor molecule forming a membrane-bound form [12]. Throughout embryonal development, expression of TNFα together with TNFβ is constitutive, and therefore the cytokine network can be formed by TNFα and TNFβ as initiator molecules.

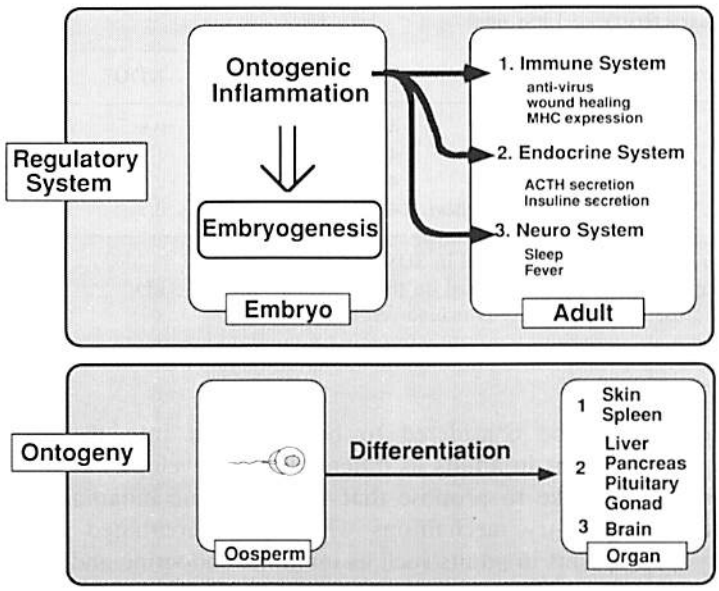


Fig. 4. Ontogenic inflammation as a prototype of regulatory mechanism in the adult.

Ontogenic Inflammation as a Prototype of Regulatory Mechanisms in the Adult

As we observed a clue that ontogenic inflammation actually plays a key role in embryonal development, the idea that it can be regarded as the prototype of regulatory mechanisms such as immune, endocrine, and neurobiological systems is quite persuasive. Thus, our new concept as demonstrated in figure 4 is that ontogenic inflammation can be regarded as a prototype of regulatory mechanisms in adult. Ontogenic inflammation can be differentiated into regulatory mechanisms in adults, in which leukocytes, especially macrophages, play a key role. The whole process of this development and differentiation corresponds well to that of the morphological differentiation.

As shown in figure 4, a vast knowledge of biological functions of activated macrophages or TNFα revealed that they play a key role for regulation in various phenomena involved in immune, endocrine and neurobiological systems in adults [17–23]. On the other hand, oosperm differentiated into many organs which play different functions in adults. Therefore, ontogenic inflammation should be a mighty regulatory mechanism in embryogenesis, being a prototype of regulatory mechanism in

Table 1. Characteristic of LPSs used

Abbreviation	Origin	Molecular size[a]	P[b]	KDO[b]	Hexosamine[b]
LPSe	*Escherichia coli*	4–15, 30–50	2	0.8	5
LPSe	*Bordetella pertussis*	4–5	3	1	10
LPSp	*Pantoea agglomerans*	4–8, 30–35	2	2	2
LPSw	*Triticum aestivum* (wheat)	4–6	0.5	0.7	3.5

[a] Molecular size of LPSs as observed in SDS-PAGE.
[b] Each composition as expressed based on the molecular ratio per 5 kD.
P = Phosphorus; KDO = 3-deoxy-D-manno-octulosonic acid.

adults, and it can be considered to be converted into those diverse regulatory mechanisms in adults as differentiation develops.

Now I should like to propose that the ontogenic inflammation is a prototype of regulatory mechanisms which is differentiated to diverse regulatory mechanisms in adults such as immune, endocrine and neurobiological systems. Once diseases occur in adults, reproduction of ontogenic inflammation should restore the homeostasis to cure the diseases.

Together with these new findings in fundamental research mentioned above, we have carried out experiments to prove whether activation of macrophages can restore various intractable diseases based on the idea shown in figure 4 by the use of LPS of small molecular size [2–10]. We thought that development of our new concept shown above is to lead a new therapy for various intractable diseases by a single event, activation of macrophages up to the stage of ontogenic inflammation.

LPS of Small Molecular Size as One of Inducers of Ontogenic Inflammation in the Adult

We use three kinds of LPS of small molecular size (table 1). Their physiological and biological properties have been examined in detail [2, 3]. We named extracts from wheat flour, LPSw, LPS from Bordetella, LPSb, LPS from *Pantoea agglomerans* which was found in any sort of wheat flours, LPSp. Details have been given by Nishizawa et al. [24] and Tsukioka et al. [25].

We have already reported that LPS of small molecular size can induce ontogenic inflammation in adults, and its therapeutic usefulness, such as protective effects on gastric ulcer [4], pain relief of rheumatism [5], curative effects on type I diabetes mellitus [6], protective effect on acute infection of

Toxoplasma gondii [7], curative and preventive effect in hyperlipidemia [8], possible preventive effect on osteoporosis [9, 10] administered orally or cutaneously. Activated macrophages in the state containing precursor TNFα (primed stage) can be considered to show these effects.

Ontogenic Inflammation as a Prototype of Regulatory Mechanism in the Immune System

I would like to refer to prevention of bacterial infection or viral infection as the activity mainly ascribable to immune systems. For example, either LPSw or LPSb in various dosages were administered ad libitum in drinking water to mice for 3 weeks. They were then challenged with bacteria in the peritoneal cavity at a dose of 5×10^5 CFU of *Salmonella typhimurium*, which is nearly a lethal dose. Two or 20 ng/ml of LPSb (fig. 5a) could significantly prevent mice from bacterial infection analyzed by survival ratio. Interestingly, 200 ng/ml of LPSb decreased survival ratio compared with 2 or 20 ng/ml of LPSb. Similar effects were also observed with LPSw (fig. 5b). Such low doses of LPSw or LPSb could not produce free TNF in mice. On the other hand, oral administration of small molecular size of LPS was shown to activate macrophages, and we consider that these preventions can be due to induction of ontogenic inflammation.

The following experiment was carried out to test the preventive effect of LPSb or LPSw administered orally or cutaneously on viral infection in mice. Various amounts of LPSw or LPSb were administered ad libitum in drinking water for 3 weeks before viral infection. Mice were then challenged with LD_{60} of Aujeszky's virus which belongs to the herpes virus group in the peritoneal cavity. The survival ratio of 2–20 ng/ml in LPSw or LPSb was significantly higher than that of the controls (fig. 6). Antiviral antibody was not detected in all surviving animals treated with LPS. Therefore, viral infection might be inhibited by treatment of LPSw as well as LPSb.

Intranasal administration of LPSb was also preventive against viral infection. On one occasion, administration of 10 μg/ml of LPSb revealed quite a strong antiviral activity analyzed by survival ratio (fig. 7). LPSb might be absorbed through the intranasal mucosa and then activates macrophage-like cells under the mucosa.

From these results, LPS of small molecular size can be administered orally or percutaneously without harm. Both experiments demonstrate that LPSb or LPSw acts as a potentiator of immune function through activation of macrophages. Thus it is suggested that ontogenic inflammation as a prototype might be involved in the regulation of the immune system.

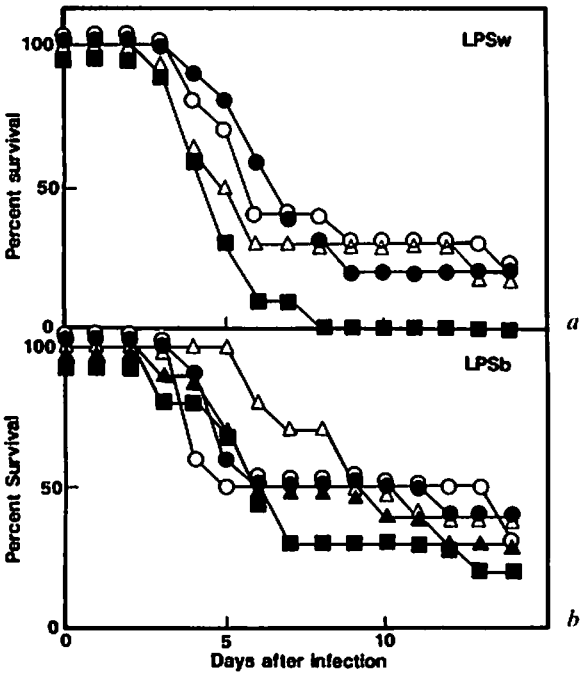


Fig. 5. Preventive effect of oral administration of LPSw and LPSb ad libitum on Salmonella infection. *a* C57BL/6 mice (n = 10) administered LPSw at concentrations of 2 (○), 20 (●) or 200 ng/ml (△) respectively in drinking water from 3 to 8 weeks of age. Control mice (■) received distilled drinking water. A challenge infection was performed by intraperitoneal injection of 4×10^5 CFU *S. typhimurium* at 6 weeks of age. *b* C57BL/6 mice (n = 10) administered LPSb at concentrations of 0.2 (○), 2 (●), 20 (△) or 200 ng/ml (▲) respectively in drinking water from 3 to 8 weeks of age. Control mice (■) received distilled drinking water. A challenge infection was performed by intraperitoneal injection of 5×10^5 CFU *S. typhimurium* at 6 weeks of age.

Ontogenic Inflammation as a Prototype of Regulatory Mechanism in the Neuro-Endocrine System

We revealed that LPS showed analgesic effects if administered orally, as also shown by Inagawa et al. [26], and suggested that this effect might be due to secretion of β-endorphin. First, we analyzed whether LPSp can induce β-endorphin or not. β-Endorphin was actually induced by intravenous administration of LPSp (fig. 8a). Kinetics of production of β-endorphin coincide well with analgesic effects (fig. 8b). These results prompted us to examine whether or not morphine addiction can be

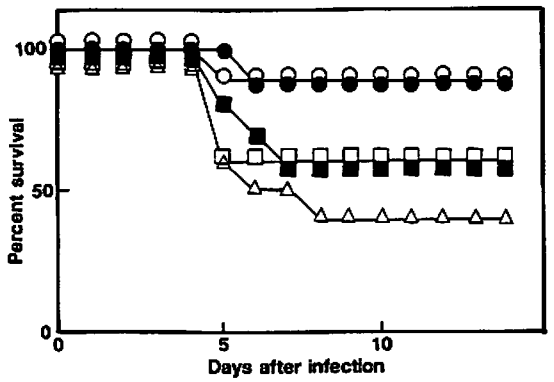


Fig. 6. Preventive effect of intranasal administration of LPSb on Aujeszky's virus infection. C57BL/6 mice (n = 10) administered LPSw at concentrations of 2 (○), 20 (●) or 200 ng/ml (□) respectively, or 20 ng/ml of LPSb (△) in drinking water from 3 to 8 weeks of age. Control mice (■) received distilled drinking water. A challenge infection was performed by intraperitoneal injection of Aujeszky's virus ($10^{2.1}TCID_{50}$).

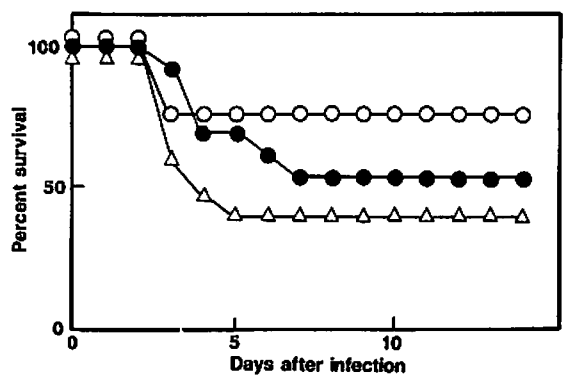


Fig. 7. Preventive effect of oral administration of LPSw and LPSb ad libitum on Aujeszky's virus infection. C57BL/6 mice (n = 13–15) were intradermally inoculated with Aujeszky's ($10^{2.1}TCID_{50}$) at 6 weeks of age. One day after the inoculation, mice were intranasally administered LPSb at the doses of 10 μg/mouse (○), 100 μg/mouse (●) or vehicle (△), respectively.

restored by LPSp, because β-endorphin is known as an intrinsic opioid that acts as a morphine-like substance.

We used naloxone-induced jumping in mice for analysis of LPS function against morphine dependence [27, 28]. Only 10 μg/mouse, namely 500 μg/kg of intradermally administered LPSp, revealed the strongest

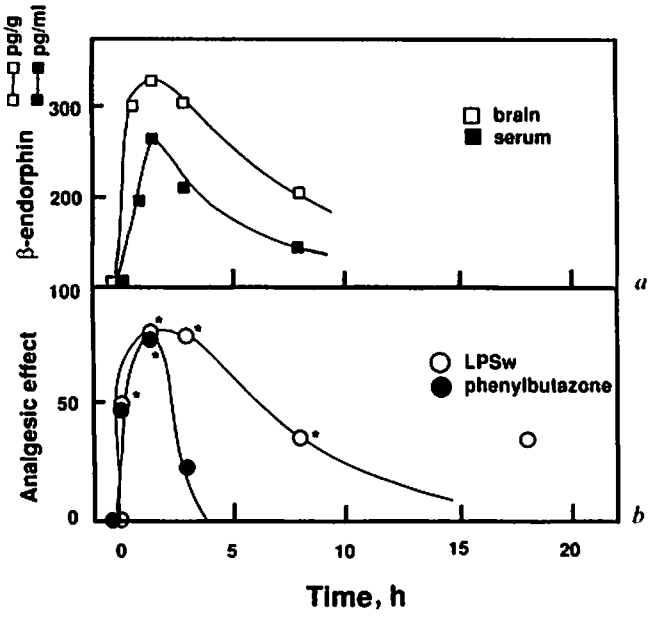
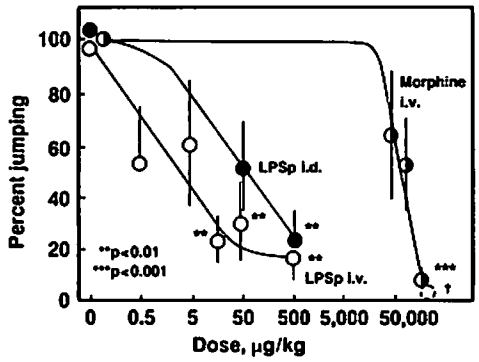


Fig. 8. Time course of production of β-endorphin after administration of LPSp. *a* Time course of production of β-endorphin. 10 μg of LPSw was injected into C3H/He mice (n = 2) intravenously. 0–8 h thereafter, mice were bled to obtain serum and brain tissue. The brain tissue was homogenized and then centrifuged to obtain the supernatant. β-endorphin in both brain homogenate (□) and serum (■) were assayed by RIA. The shaded area shows β-endorphin levels of normal mice in the brain and serum. *b* Time course of analgesic effect of LPSw. Analgesic effect was evaluated by an acetic acid induced writhing syndrome test. 1 μg LPSw (○) or 1 mg phenylbutazone (●) was injected into C3H/He mice (n = 6) intravenously. 0–18 h after the administration, 0.5 ml and 1% acetic acid solution was injected intraperitoneally and the number of stretch movements counted for 30 min. Analgesic effect was expressed by percent inhibition as compared with that in the saline-injected group as control. *$p < 0.01$ (by Student's t-test).

activity for morphine dependence (fig. 9a). Other conventional drugs for morphine dependence, such as clonidine or morphine itself, are also effective for relief of morphine dependence. However, an effect comparable to that of 10 μg of LPSp can be obtained nearly at a lethal dose (fig. 9b). In addition to inhibition of jumping, we found that LPSp could prevent weight loss without harm which was one of the most serious problems accompanied with morphine addiction. As to the mechanisms for relief of morphine dependence by LPSp, we considered that neuroendocrine function should play a key role in restoring morphine dependence through activation of macrophages, since we confirmed that TNF could also exert preventive



a

	Therapeutic index (LD_{50}/ED_{50})
LPSp	220
Morphine	4
Clonidine	no effect

b

Fig. 9. a Effect of intravenous or intradermal injection of LPSp and morphine on jumping action during naloxone-precipitate withdrawal. Male ddY strain mice (n = 5–10) were used. They were made morphine-dependent by the implantation of a pellet containing 12.7 ± 1.0 mg morphine hydrochloride. After 48 h, LPSp was injected intravenously (○) or intradermally (●), 2 h later, naloxone hydrochloride (10 mg/kg) was injected intraperitoneally and then the number of jumps was counted for 40 min. Morphine hydrochloride was injected intravenously (◑) and 20 min later, naloxone hydrochloride was injected intraperitoneally. *b* The percentage of jumps was calculated as the ratio of the number of jumps of the LPSp or morphine injected mouse to that of the saline-injected mouse.

effects of jumping [27]. These effects are induced through the same mechanisms of relief of pain, namely β-endorphin induction [for details, see 28].

We have previously reported the pain-relieving activity of LPS against postherpetic neuralgia, and report further data concerning the pain-relieving activity of acute herpes patients with HIV infection or systemic lupus erythematosus as underlying diseases. Pain relief was attained in 7 patients within 24 h after LPSw administration (table 2a). More than 70% of the treated patients showed quite good results. No adverse reaction or side effects related to the use of LPSw were observed (table 2b). Most of them had underlying diseases by which immune function might be more or less distorted. These results suggested that the effectiveness of LPSw may be due to the activation of the neuroendocrine system together with that of the immune system. In addition to pain-relieving activity, the healing process of blisters seemed to be accelerated.

Table 2. A list of patients (*a*) with localized herpes zoster (HZ) treated with LPSw and (*b*) clinical efficacy of LPSw

a

Patient	Age/ sex	Underlying disease	Lesion	Onset of HZ	Start of LPSw	Concurrent start of aciclovir	Additional use of aciclovir	Blister	Pain relief[a]
1 K.T.	41/F	HIV (ARC)	lt leg	9/28	10/5	–	–	improvement	G5/G2
2 Y.S.	14/M	HIV (AC)	lt chest	1/6	1/7	–	+	progress	G5/G3
3 M.A.	15/F	SLE	lt chest	3/24	3/28	–	+	progress	G5/G3
4 K.Y.	47/F	SLE	lt chest	4/1	4/2	–	–	no change	G5/G1
5 T.A.	25/M	CML	rt face	4/15	4/30	–	–	–[b]	G5/G3
6 H.S.	69/M	lung Ca	rt chest	4/29	4/30	–	+	progress	G4/G4
7 F.T.	24/M	–	rt chest	6/17	6/19	+		no change	G4/G3
8 T.S.	40/M	HIV (AIDS)	rt face	6/20	6/25	+		no change	G1/G1
9 K.N.	27/M	HIV (ARC)	rt chest	8/22	8/24	+		no change	G5/G3
10 O.S.	37/M	–	rt chest	9/1	9/2	+		no change	G5/G3

b

Clinical efficacy of LPSw[c]

excellent	good	fair	poor
2	5	1	2

Ten patients who had localized HZ and had been hospitalized at the Institute of Medical Science, University of Tokyo, were enrolled. As underlying diseases, HIV (human immune deficiency virus) was infected, SLE (systemic lupus erythematosus), CML (chronic myelogenous leukemia), lung Ca (lung carcinoma); 1 μg/ml of LPSw in 50% glycerol solution was used. It was applied to the local lesions 5–6 times/day and was continued for 7 days.

Pain relief was evaluated 24 h after the LPS treatment. Pain grade is classified as follows: G0 = no pain; G1 = almost no pain; G2 = slight pain but not continuous; G3 = slight pain but continuous; G4 = severe pain, analgesics are not necessary; G5 = severe pain, analgesics are necessary.

[a] Pain grade before/after 24 h.

[b] Postherpetic neuralgia.

[c] Criteria is as follows: excellent = improvement of pain, more than 3 pain grades; good = improvement of pain, more than 2 pain grades; fair = improvement of pain, more than 1 pain grade; poor = no change.

Apart from the examination of our new concept, the clinical usefulness of LPS of small molecular size is now summarized. The first advantage of LPS of small molecular size is that it can be administered orally or percutaneously, and therefore no adverse side effects such as fever or septic shock were observed after more than 100 cases. Second, since the effectiveness of LPS of small molecular size can be ascribed to its function to induce the precursor TNF in macrophages, that is to say to provoke the ontogenic

inflammation, it is no wonder that many intractable diseases are cured by a single drug, LPS of small molecular size. Its precise clinical results in herpes zoster patients have been discussed by Goto et al. [29]. Separately, the usefulness of endogenous TNF against refractory tumors has also been discussed by Takagi et al. [30] Oya et al. [31] and Noguchi et al. [32]. Clinical trials against other intractable diseases are needed and we are now in the process of conducting such trials.

However, to return to ontogenic inflammation again: From all our new findings presented, I would like to stress again our hypothesis that ontogenic inflammation should be regarded as a prototype of regulatory mechanisms in the adult, and based on this idea, namely provocation of ontogenic inflammation through activation of macrophages, as we have done with LPS, will lead to new clinical approaches to various diseases.

Mechanism of Provocation of Ontogenic Inflammation by LPS Administered Orally or Percutaneously

The practical usefulness of LPS of small molecular size against various intractable disease is promising. However, the precise mechanism to activate macrophages by use of oral or percutaneous administration of the LPS still remains to be solved. The possible mechanism for activating macrophages by LPS administered orally or percutaneously is now proposed (fig. 10). Since we could not detect any LPS in serum after oral or percutaneous administration of LPS at doses of more than 1 mg, it may act indirectly to provoke ontogenic inflammation. Thus, administered LPS can enter the dermis through sweat pores and/or hair follicles of skin or sutured lesions, and then activate histiocytes or macrophages on the spot. Activated cells may enter the bloodstream and reach the suitable part to play a key role in restoration of homeostasis.

Distribution of LPS of Small Molecular Size in Various Plants

A variety of materials from plants contain significant amounts of LPS (table 3). Therefore, our homeostasis can possibly be maintained partly by the uptake of such materials without knowing about LPS. On the other hand, LPS is not found in the animal kingdom. LPS is also an absolutely foreign substance to all animals. However, according to the concept of ontogenic inflammation, an intrinsic factor to provoke initial ontogenic inflammation, for example at early embryonal development before the morula stage, should exist in the animal body, because, during embryonal

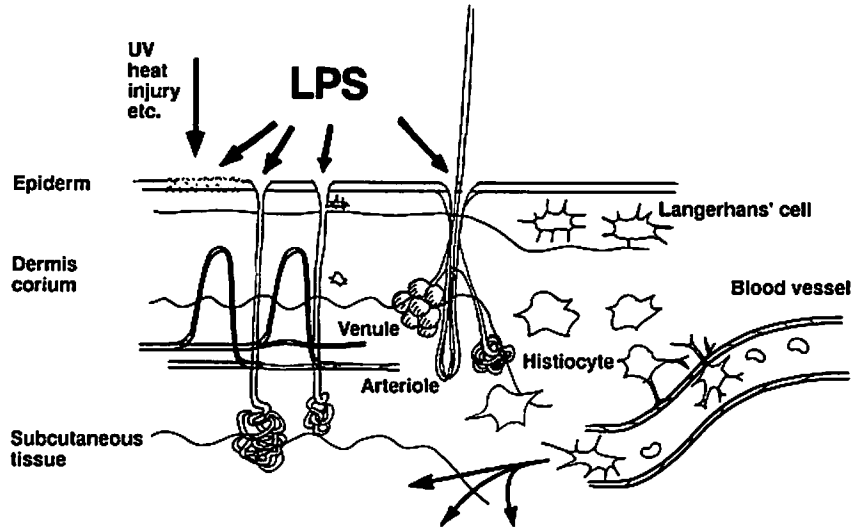


Fig. 10. Hypothesis of macrophage activation by percutaneous administration of LPS of small molecular size.

development, especially in mammals, a foreign substance like LPS could not enter the embryo. In this regard, we found that some sort of gangliosides could act in the same manner as LPS which can activate macrophages through activation of complement pathway [Oshima et al., unpubl. data].

Conclusion

Based on ontogenic inflammation at the molecular level, we can reach the innovative concept that ontogenic inflammation should be a prototype of regulatory mechanisms in adults. In adults, responsible cells to initiate ontogenic inflammation are restricted to macrophages with precursor TNF molecule on its membrane surface. This stage might be equal to macrophages activated by MAF. Actually, macrophages are widely distributed in many organs at the resident state. When we hypothesize ontogenic inflammation as a prototype of regulatory mechanisms in adults, we can find further insight into the significance of macrophages concerning the central role in the regulatory system for maintaining homeostasis. Divergent biological effects or function of the various cytokines known in immunology, endocrinology, neurobiology and the regulatory system in adults can be clearly arranged and understood by ontogenic inflammation.

Table 3. LPS content in the various plant samples

LPS μg/g	Dicotyledoneae	Monocotyledoneae	Other plants
1,000	*Sinomenium acutum* Rehd. et Wils. (600)		*Chlorella* (2,500)[1]
			Undaria pinnatifida suringar (400)
			Cordyceps sinensis Berk. Sacc. (240)
		Curcuma domestica Valeton (turmeric) (200)	(Kelp) (240)
			Grifola frondosa (210)
			Asakusa lavor (130)
100	*Gynosfemma pentaphyllum* Thumb. Makino (73)		*Hijikia fusiformis* (85)
	Seseli libanotis Koch (50)		Jew's ear (80)
			Lentinus edodes Sing., *Cortinellus shiltake* P. Henn. (40)
	Panax ginseng C.A. May (45)		*Lyophyllum shimeji* (40)
		Zingiber mioga (41)	Winter mushroom (40)
	Actinidia polygama Maxin (40)		
			Pholiota nameko (20)
	Lyopersicon esculentum (tomato) (11)	*Sasa albo-marginata* (15)	Mushroom (20)
	Cucurbita moschata (pumpkin) (10)		(Royal fern) (10)
10	*Citrus aurantium* (8)		
	Uncaria rhynchophylla Miquel (7)	*Pinellia ternata* Breitenbach (6)	
	Prunus persica L. (peach) (5)	Asparagus (5)	
	Pueraria lobata Ohwi (3)	*Ophiopogon japonicus* Ker-Gawl. (4)	
	Piper nigrum (pepper) (2)	*Triticum aestivum* L. (wheat) (4)	
	Capsicum annuum L. (2)	*Iris sanguinea* (3)	
	Juglans regia (walnut) (2)	*Oryza sativa* (rice) (1)	
	Avocado (1)		
1	Japanese medlar (0.8)		
	Broad bean (0.8)		
	Soy bean (0.2)	*Zea mays* L. (corn) (0.2)	
		Pinus spp. (0.1)	
		Allium sativum L. (garlic) (0.07)	

All samples were washed with distilled water, dried up to a constant weight by a heating dryer and vacuum desiccator. After mincing, 1 g of powdered sample was suspended in 5 ml of distilled water and heated for 5 h at 60 °C for extraction of LPS. After extraction, the supernatant was obtained by centrifugation (at 2,000 g for 20 min). LPS contents in samples were measured by Toxicolor (a kit preparation of conventional Limulus test) and Endospecy kit (without G-factor).

[1] *Chlorella* preparation free of bacteria shows completely negative.

Acknowledgements

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