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Macrophage Activation by an Ornithine-Containing Lipid or a Serine-Containing Lipid

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α-*N*-(3-Acyloxyacyl)-ornithine (or -serine) is the structure of lipoamino acids obtained by us previously from some gram-negative bacteria (Y. Kawai and I. Yano, Eur. J. Biochem. 136:531–538, 1983; Y. Kawai, I. Yano, and K. Kaneda, Eur. J. Biochem. 171:73–80, 1988; Y. Kawai, I. Yano, K. Kaneda, and E. Yabuuchi, Eur. J. Biochem. 175:633–641, 1988). The 3-acyloxyacylamide structure is present in both the lipoamino acids and lipid A of lipopolysaccharide (endotoxin). The efficacy of lipoamino acids (an ornithine-containing lipid and a serine-containing lipid) in activating C3H/HeSlc mouse peritoneal exudate macrophages was compared with that of bacterial lipopolysaccharide, because the two types of substances were expected to exhibit similar biological activities and physiological functions on the basis of their structural similarities. Actually, the lipoamino acids, as well as lipopolysaccharide, strongly activated the macrophages to generate the immunoregulatory substances prostaglandin E_2 and interleukin-1, but their effect on the induction of L929 cell cytolytic factor (a possible tumor necrosis factor), another immunoregulatory substance, was weaker than that of lipopolysaccharide. The effect of lipoamino acids on the cytotoxicity of macrophages for EL-4 leukemia cells was very weak. However, all of these activities, as far as tested, were strongly enhanced by synergistic action with gamma interferon. Only the serine-containing lipid killed both C3H/HeSlc and C3H/HeJ macrophages to almost the same degree as endotoxin killed C3H/HeSlc macrophages. On the other hand, lethal toxicity for mice was not found with either the ornithine-containing lipid or the serine-containing lipid, even when 7 mg of compound was injected into a mouse. These studies suggest that the lipoamino acids are nontoxic characteristic immunoactivators.

Lipoamino acids such as an ornithine-containing lipid and a serine-containing lipid, as well as lipopolysaccharide (LPS) (endotoxin) of gram-negative bacteria and lipoteichoic acid of gram-positive bacteria, are amphipathic substances of bacterial origin. In the last few years, Kawai and colleagues purified some lipoamino acids from *Bordetella* (14–18), *Flavobacterium* (19), *Pseudomonas* (20), and *Achromobacter* (14) species and determined that their structures were α-*N*-(3-acyloxyacyl)-ornithine or -serine (Fig. 1). Although ornithine-containing lipids are found in various bacteria (14, 18–21, 39), serine-containing lipids obtained from *Flavobacterium* species (19) are very rare. Liposomes of lipoamino acids agglutinate human and rabbit erythrocytes strongly at a concentration of 1 μg/ml, mainly by lipid interaction (18). The hemagglutination is specifically inhibited by phosphatidylcholine (13, 17, 18). The carbon chain length required for both central and terminal fatty acids of lipoamino acids to cause the strong hemagglutination is believed to be 3-OH $C_{16:0}$ linked to $C_{16:0}$ or 3-OH $C_{17:0}$ linked to $C_{15:0}$.

Bacterial LPS (endotoxin), its lipid A moiety (11, 22, 28, 33–35), and synthetic lipid A analogs (10, 11, 22, 33, 38) are well known to induce macrophages to generate immunoregulatory substances, including tumor necrosis factor, prostaglandins, interleukin-1 (IL-1), interferons, and colony-stimulating factor. The lipoamino acids are structurally similar to the parts of lipid A which are responsible for the endotoxic activities of LPS in the amide and ester linkages of fatty acids (Fig. 1). From this viewpoint, LPS-like biological activities and physiological functions of lipoamino acids are expected. On the other hand, lipoamino acids are different from lipid A with respect to the hydrophilic part of the structure (amino acid in the former and glucosamine in the latter), although both types of substance are amphipathic. For this reason, some activities of lipoamino acids different from those of LPS are expected, too.

In this paper, we compare macrophage activation by lipoamino acids with that by LPS in terms of the relationship between chemical structure and biological activities. We suggest that the lipoamino acids represent a new kind of immunoactivator.

MATERIALS AND METHODS

Lipoamino acids. The lipoamino acids used here were a type II ornithine-containing lipid and a serine-containing lipid prepared from *Flavobacterium meningosepticum* (19); their structures are shown in Fig. 1. Just before use, the liposomes of the lipoamino acids were prepared as described previously (18).

Reagents. Phenol-extracted LPS from *Escherichia coli* O111:B4 was obtained from Sigma Chemical Co., St. Louis, Mo. Recombinant murine gamma interferon (IFN-γ) was kindly provided by Toray Industries, Tokyo, Japan, and was used at a concentration of 10 U/ml in all the experiments.

Culture medium. RPMI 1640 medium was prepared from powdered stock (Nissui Seiyaku Co., Ltd., Tokyo, Japan) and was supplemented with 100 U of penicillin G potassium (Banyu Seiyaku Co., Ltd., Tokyo, Japan) and 100 μg of streptomycin sulfate (Meiji Seika Co. Ltd., Yokohama, Japan) per ml. Fetal bovine serum (FBS) (GIBCO Laboratories, Grand Island, N.Y.) was inactivated at 56°C for 30 min before use.

Mice. Specific-pathogen-free C3H/HeSlc, C3H/HeJ, and ICR/Slc mice, 6 weeks old, were obtained from Shizuoka

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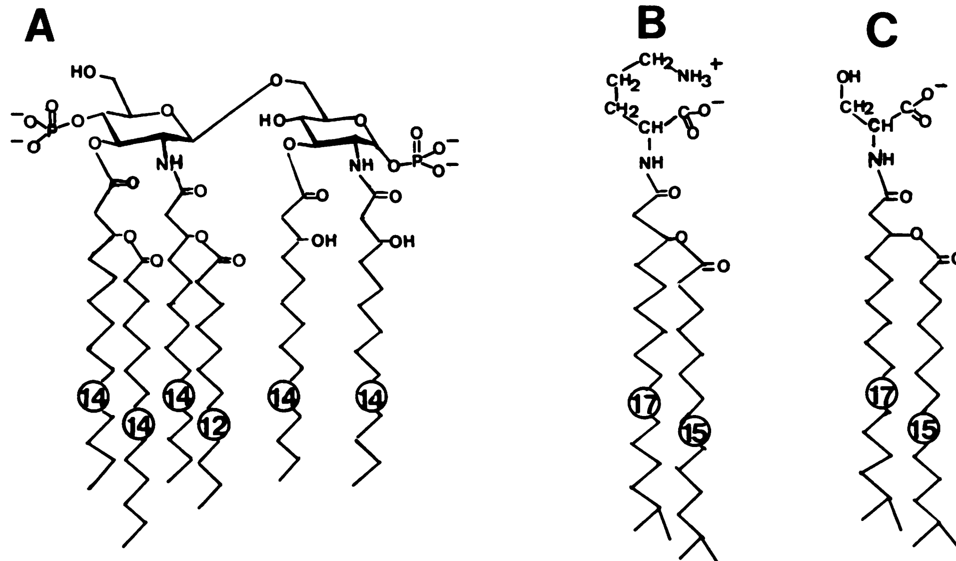

FIG. 1. Structures of the ornithine-containing lipid (B) and the serine-containing lipid (C) of *F. meningosepticum* and of *E. coli* lipid A (A).

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Preparation of macrophages. Proteose peptone- or Brewer thioglycolate medium (Difco Laboratories, Detroit, Mich.)-induced peritoneal exudate macrophages (P-PEM and T-PEM, respectively) were prepared as described previously (1–3). The purity of the macrophages thus prepared was greater than 96%, according to morphologic and phagocytic criteria.

Production of a factor cytolytic for L929 cells (a possible tumor necrosis factor). After T-PEM adhering to a 24-well plate (10^6 cells per well) were incubated at 37°C for 20 h with 1 ml of RPMI 1640 medium containing FBS or supplemented with IFN-γ, they were washed and incubated for a further 20 h with lipoamino acids (25 to 200 μg/ml) or LPS (1 to 10 μg/ml). The culture supernatants were harvested and stored at −20°C until use.

Assay for the L929 cell cytolytic factor. The assay was carried out by the method of Drysdale et al. (9). L929 cells (3 × 10^4 per well) overlaid with both 0.1 ml of twofold-diluted test sample and 0.1 ml of RPMI 1640 medium containing actinomycin D (5 μg/ml) in a 96-well plate were incubated at 37°C for 24 h. After removal of the supernatants, the remaining cells were stained with 0.5% crystal violet solution for 15 min, rinsed with water, and dried. The A_{750} of the dye released from the stained, unlysed cells into 1% sodium dodecyl sulfate solution was determined by using a model ER-8000 Sjeia Autoreader (Sanko Junyaku Co., Ltd., Tokyo, Japan). Cytolytic activity for L929 cells was expressed as the dilution of the sample causing 50% cell lysis (1 unit).

Assay for PGE$_2$. P-PEM adhering to a plate (10^6 cells per well) were incubated at 37°C for 20 h with 1 ml of lipoamino acids (25 to 100 μg/ml) or LPS (10 μg/ml) in the presence or absence of IFN-γ. The culture supernatants were harvested for the prostaglandin E_2 (PGE$_2$) assay. The amount of PGE$_2$ in the culture supernatants was determined with a PGE$_2$ ^{125}I radioisotope assay kit from Dupont, NEN Research Products, Boston, Mass.

Assay for IL-1. The IL-1 assay was carried out as described by Mizel et al. (24). C3H/HeJ mouse thymocytes (5 × 10^5) suspended in 0.1 ml of RPMI 1640 medium containing 10% FBS and 5 × 10^{-5} M 2-mercaptoethanol in each well of a 96-well plate were incubated at 37°C for 96 h with 50 μl of concanavalin A (Sigma) (0.2 μg/ml) and 50 μl of the culture supernatants prepared for the L929 cell cytolytic factor assay. The cells were pulsed with 0.5 μCi of [^{3}H]thymidine per well during the last 18 h of culturing. At the end of the culture period, the cells were harvested on glass fiber filters with a semiautomated sample harvester and the [^{3}H]thymidine incorporated into the cellular DNA was counted in a liquid scintillation counter. To study the effect of indomethacin (IM) on the production of IL-1, we incubated P-PEM (10^6 cells per well) at 37°C for 20 h with lipoamino acids (25 to 100 μg/ml) in the presence or absence of IM (Sigma) (5 × 10^{-6} M). The resulting supernatants were used for the IL-1 assay as described above.

Assay for cytotoxicity of macrophages for leukemia cells. The method used was described previously (2, 3). P-PEM (2 × 10^5 cells per well) were incubated at 37°C for 18 h with lipoamino acids (0.01 to 100 μg/ml) in the presence or absence of IFN-γ in 0.2 ml of culture medium containing FBS. The cells were washed, overlaid with ^{51}Cr-labeled EL-4 leukemia cells (10^4 cells per well) in 0.2 ml of RPMI 1640 medium, and incubated for a further 20 h in the presence of 5 × 10^{-6} M IM. After centrifugation of the culture plate, the released radioactivity in 0.1 ml of the supernatants was counted. Cytotoxicity was expressed as a ratio (percentage) of the experimental release of radioactivity to its maximal release.

Determination of cytotoxicity for macrophages in vitro. The experiment was performed by the method of Glode et al. (12). P-PEM adhering to a 96-well plate (2 × 10^5 cells per well) were incubated with lipoamino acids (1 to 200 μg/ml) or LPS (100 μg/ml) in the presence or absence of IFN-γ at 37°C for 40 h in 0.2 ml of RPMI 1640 medium supplemented with FBS. After the supernatants were removed, the cells were stained with 0.3% trypan blue dye in physiological NaCl solution to count the dead macrophages. Cytotoxicity for macrophages was expressed as the percentage of dead macrophages in 200 macrophages counted.

Determination of lethal toxicity for mice. C3H/HeSlc and ICR/Slc mice were used to determine whether the lipoamino acids had lethal toxicity for mice. After 1-ml quantities of the lipoamino acid liposome suspensions in phosphate-buffered

TABLE 1. Effect of lipoamino acids on the production of the L929 cell cytolytic factor in thioglycolate-induced C3H/HeSlc macrophages

Sample and concn (μg/ml)[a]	L929 cell cytolytic activity (units)	
	Without IFN-γ	With IFN-γ
Ornithine-containing lipid		
200	5	158
100	13	>256
50	9	>256
25	9	158
Serine-containing lipid		
200	2	256
100	37	>256
50	18	>256
25	<2	20
LPS		
10	16	208
1	28	>256
0.1	64	>256
0.01	18	256
0.001	<2	
Medium only	<2	<2

[a] Samples were culture supernatants (1 ml) of T-PEM (10^6 cells) incubated with lipoamino acids or LPS.

physiological NaCl solution (0.2 to 7.0 mg/ml) or LPS solution (250 to 500 μg/ml) were injected into mice intraperitoneally, the mice were observed for 1 week to determine the amount of lipoamino acids required for killing a mouse. Each experiment was carried out with three to five mice.

RESULTS

Induction of generation of the L929 cell cytolytic factor in macrophages. The amounts of the L929 cell cytolytic factor in the supernatants of T-PEM cultured with the sample were determined as described in Materials and Methods. The lipoamino acids at concentrations of 25, 50, 100, and 200 μg/ml induced generation of the factor in the macrophages, but the inducing activity was weaker than that of LPS (Table 1). The activity of the lipoamino acids was strongly increased by synergistic action with IFN-γ, as was that of LPS. The ability of the lipoamino acids to generate the cytolytic factor in C3H/HeJ macrophages was very weak, only 4 units being generated by the ornithine-containing lipid at a concentration of 100 μg/ml, even by synergistic action with IFN-γ. Experiments were always conducted three times.

Enhancement of PGE_2 generation in macrophages. The amounts of PGE_2 in the supernatants of P-PEM cultured with the samples were determined by the assay based on immunocomplex generation as described in Materials and Methods. The lipoamino acids strongly enhanced PGE_2 generation, and the activity was as high as that of LPS (Table 2). Furthermore, enhancement of PGE_2 generation by synergistic action with IFN-γ was also seen in this case. On the other hand, enhancement of PGE_2 generation by the lipoamino acids in T-PEM was very weak. Activation of C3H/HeJ macrophages to produce PGE_2 by lipoamino acids was weak; for example, the activity at the same concentration, 25 μg/ml, was 5 ng/10^6 cells.

Enhancement of IL-1 generation in macrophages. IL-1 activity in the supernatants of T-PEM cultured with the lipoamino acids was determined by thymocyte proliferation as described in Materials and Methods. The lipoamino acids activated the macrophages dose dependently to cause thymocytes to proliferate, and the efficacy of activation was high (Table 3). Although the effect of the ornithine-containing lipid on thymocyte proliferation seemed weaker than did that of the serine-containing lipid, the IL-1 activity of the ornithine-containing lipid was proved to be as almost the same as that of the serine-containing lipid when IFN-γ or IM was added at the macrophage activation step.

These results indicated that both ornithine- and serine-containing lipids stimulate macrophages to generate the immunoregulatory substances PGE_2, IL-1, and L929 cell cytolytic factor.

Effect on the cytotoxicity of macrophages for leukemia cells. The effect of the lipoamino acids on the toxicity of P-PEM for EL-4 leukemia cells is shown in Table 4. This experiment was aimed at detecting the activation of direct cytotoxicity of the macrophages, not the metabolic activation described above. A leukemia cell-toxic effect of the lipoamino acids was not demonstrated, even when they were used at a concentration of 100 μg/ml, and it was revealed only by synergistic action with IFN-γ. In this case, the efficacy of the lipoamino acids was very weak in comparison with that of LPS.

Cytotoxicity for macrophages. The killing effect of the

TABLE 2. Effect of lipoamino acids on the release of PGE_2 from proteose peptone-induced C3H/HeSlc macrophages

Sample (μg/ml)	Released PGE_2 (ng/10^6 cells)	
	Without IFN-γ	With IFN-γ
Serine-containing lipid (25)	60	150
Ornithine-containing lipid (25)	55	140
LPS (10)	49	130
Medium only	1	4

TABLE 3. Effect of lipoamino acids on IL-1 generation in thioglycolate-induced C3H/HeSlc macrophages

Sample and concn (μg/ml)	[^{3}H]thymidine uptake (cpm) (10^4)
Serine-containing lipid	
100	2.3
50	1.5
25	1.3
100 + IFN-γ	1.5
Ornithine-containing lipid	
100	1.1
50	0.8
25	0.7
50 + IFN-γ	2.2
100 + IM	1.7[a]
LPS, 10	1.1
Medium	0.6
Medium + IFN-γ	0.6

[a] This value was 0.5 × 10^4 cpm when proteose peptone-induced macrophages were tested without IM.

TABLE 4. Effect of lipoamino acids on the cytotoxicity of C3H/HeSlc macrophages for tumor cells

Sample and concn (μg/ml)	Cytotoxicity (%)	
	Without IFN-γ	With IFN-γ
Serine-containing lipid		
100	−0.3	15.3
10	1.0	6.4
1	−1.0	−4.9
0.1	0.8	2.6
0.01	0.6	−0.1
0 (Medium)	−1.3	0.7
Ornithine-containing lipid		
100	1.0	30.4
10	3.2	21.6
1	−0.4	8.8
0.1	0.7	3.6
0.01	0.8	−0.5
0 (Medium)	−1.3	0.7
LPS		
100		
10	18.7	28.4
1	8.4	26.3
0.1	9.0	30.2
0.01	6.2	25.1
0 (Medium)	−1.3	0.7

lipoamino acids for macrophages was examined (Table 5). Cytotoxicity of the serine-containing lipid for both C3H/HeSlc and C3H/HeJ macrophages was demonstrated, but the ornithine-containing lipid was not cytotoxic. The toxicity of the serine-containing lipid for both types of macrophages was as strong as that of LPS for C3H/HeSlc macrophages. It was remarkable that the serine-containing lipid was toxic for LPS-low-responder C3H/HeJ macrophages at the same intensity as for C3H/HeSlc macrophages. Additionally (Table 6), synergistic action with IFN-γ was demonstrated. For example, the killing effect of the serine-containing lipid at a concentration of 50 μg/ml was augmented 1.5 times by the addition of IFN-γ.

TABLE 5. Cytotoxicity of lipoamino acids for macrophages

Sample and concn (μg/ml)	% of the following cells that died:	
	C3H/HeSlc	C3H/HeJ
Serine-containing lipid		
200	55	40
100	23	22
50	10	12
10	2	3
1	1.5	1.5
0 (Medium)	1.5	1.5
Ornithine-containing lipid		
200	5.5	2.5
100	3.0	1.5
50	2.0	
10	1.5	
1	1.5	
0 (Medium)	1.5	1.5
LPS		
100	21	5

TABLE 6. Effect of IFN-γ on the cytotoxicity of lipoamino acids for C3H/HeSlc macrophages

Sample and concn (μg/ml)	% Dead cells	
	Without IFN-γ	With IFN-γ
Serine-containing lipid		
200	61	61
100	56	55
50	30	45
25	14	22
12.5	2	8
0 (Medium)	1.0	1.5
Ornithine-containing lipid		
200	3	10
100	2	6
50	2	4
25	2	3
12.5	0.5	3
0 (Medium)	1.0	1.5
LPS		
100	17	40

Lethal toxicity for mice. The lipoamino acids were tested for lethal toxicity for mice. When 0.2, 0.5, 1, 4, or 7 mg of the ornithine-containing lipid or the serine-containing lipid was injected into mice intraperitoneally, none of the mice died, although about 60% of the mice died when 250 μg of LPS was injected. No toxicity of the lipoamino acids for animals was definitely demonstrated.

DISCUSSION

We have compared the biological activities and physiological functions of lipoamino acids with those of LPS, because the two types of amphipathic substance resemble each other somewhat in chemical structure. The lipoamino acids, as well as LPS, were shown to stimulate murine macrophages to generate the immunoregulatory substances PGE_2, L929 cell cytolytic factor, and IL-1. These immunoregulatory substances not only contribute to maintaining homeostasis in the living body but also cause the fever and lethal shock of endotoxemia (4–8, 25, 27, 30, 33–35). Furthermore, PGE_2 is related to some physiological functions, including abortion and arterial hypotension (34). The ability of the lipoamino acids to stimulate macrophages to generate PGE_2 and IL-1 was as high as that of LPS, but the effect of the lipoamino acids on the generation of the L929 cell cytolytic factor and leukemia cell cytotoxicity was weaker than that of LPS. Even when the pathway of PGE_2 generation was blocked by IM (5×10^{-6} M), the production of the L929 cell cytolytic factor was not increased much (unpublished data). Recently, Takada et al. presented the classification of biological activities of lipid A as categories I to III on the basis of structural requirements (37), although this classification had been suggested by many other researchers (10, 11, 22). The fact that the induction of the L929 cell cytolytic factor by the lipoamino acids was lower than that by LPS seems reasonable in view of the classification of a high structural requirement (category II-1) for the activity. In addition, it is interesting that lipoteichoic acid also exhibited tumor necrosis factor-inducing activity (L929 cell cytolytic activity) (40), although its chemical structure is different from that of lipid A (in polar head carbohydrate structure and fatty acid linkage mode). Although the fact that the enhancement of

IL-1 generation by the lipoamino acids was comparable to that by LPS agreed with the classification of a lower structural requirement for the activity, the fact that the enhancement of PGE_2 generation by the lipoamino acids was as high as that by LPS suggested their unique mechanism of macrophage activation and reflected their structural specificity. In molecular size, the lipoamino acids correspond to the monosaccharide derivatives of lipid A, whose biological activities are weak in general (23, 28, 32, 36, 38). The lipoamino acids, however, strongly enhanced PGE_2 and IL-1 generation. On the basis of these studies and considerations, we resolved that the lipoamino acids are a new kind of immunoregulatory substance which demonstrate a characteristic activity pattern different from that of lipid A and related substances.

On the other hand, the lipoamino acids, as well as the monosaccharide derivatives of lipid A (23, 31, 38), were not lethally toxic for mice. The ornithine-containing lipid was not cytotoxic for macrophages, but the serine-containing lipid was somewhat cytotoxic (Table 5). The lower lethal toxicity of the lipoamino acids for mice may arise from their lower ability to induce macrophages to generate the L929 cell cytolytic factor, which is presumed to be tumor necrosis factor. It is noteworthy that macrophage activation by the lipoamino acids was generally enhanced by synergistic action with IFN-γ, as was that of LPS (1, 3). It was presumed that the binding to and action on the macrophages of the ornithine-containing lipid were not uniform and were not sufficiently strong because the formation of the liposomes of the ornithine-containing lipid is not as good as that of the serine-containing lipid. This presumption was definitely indicated in the case of the activation of IL-1 generation. Namely, the serine-containing lipid could activate macrophages to generate IL-1 without IFN-γ, but the ornithine-containing lipid needed the help of IFN-γ for activation. The action of the serine-containing lipid on the cells may in fact be inhibited by the presence of IFN-γ. Furthermore, as we stated at the Annual Meeting of the Japanese Society for Bacteriology in 1987 and at the International Symposium on Endotoxins in 1988 at Jichi Medical School (manuscript in preparation), lipoamino acids exhibited B-lymphocyte mitogenicity for LPS-responder C3H/HeSlc and LPS-low-responder C3H/HeJ mice that was as strong as the activity of LPS for LPS-responder C3H/HeSlc mice.

The lipoamino acids were shown to activate macrophages to generate PGE_2, IL-1, and L929 cell cytolytic factor and not to be lethally toxic for mice. In addition, B-lymphocyte mitogenicity and macrophage-mediated cytotoxicity of the lipoamino acids for EL-4 leukemia cells were demonstrated. Furthermore, the lipoamino acids can be model substances for LPS studies, because they exhibit similar strong immunoactivation despite their small molecular size.

Finally, the lipoamino acid preparations were of high purity; therefore, the problem of contamination with LPS hardly needs consideration. The lipoamino acids were purified from the neutral solvent-extractable lipid fraction of the bacteria, but lipid A was obtained from the residual bound lipid fraction as described previously (15, 18). In addition, some biological activities and physiological functions of lipoamino acids distinctive from those of LPS were demonstrated in these studies. To make sure of the immunological activities of lipoamino acids, we carried out a limulus test by the method of colorimetric endotoxin determination (26, 29). The reaction-positive substance (0.1 ng/ml) was detected in the lipoamino acid sample (10 μg/ml). The macrophage activation by the lipoamino acids proved in this paper was not affected by such a small amount of contaminating LPS, even if the colored substance was LPS. Furthermore, the possibility of a cross-reaction between the lipoamino acids and LPS may be considered.

On the basis of these investigations it was concluded that some activities of the lipoamino acids, as described in this paper, were derived from the lipoamino acids themselves.

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