INTERACTIONS BETWEEN BACTERIAL PYROGEN AND PROTEOLIPID EXTRACTED FROM THE CEREBRUM (III)
VARIATION IN AFFINITY OF PROTEOLIPID PROTEINS DERIVED FROM RABBIT, RAT AND CHICKEN CEREBRUMS TO BACTERIAL PYROGEN

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Abstract—We undertook comparative studies on the binding of cerebral proteolipids to LPS using rabbits, rats and chickens in order to clarify the differences in inactivating effects of proteolipids from these three sources on the pyrogenicity of LPS. The Sephadex LH-20 column elution profiles of lipid phosphorus and cerebrosides were not significantly different for the three sources of proteolipid, but a larger amount of proteolipid-protein possessing LPS pyrogenicity inactivating potency was eluted with chloroform(C)/methanol(M) (4:1) in rats and chickens than in rabbits. A complex of proteolipid-protein with LPS was obtained in C effluent from incubation mixtures of rat and chicken proteolipids with LPS, corresponding to our previous observation with rabbit proteolipid. The increasing order of binding capacity of the proteolipids derived from the three species was as follows: chicken, rat and rabbit, which was in parallel with pyrogenic inactivating potency. From these data, we suggest that the difference in pyrogenic inactivating potency among the proteolipids of some species depends on the difference in binding capacity of the proteolipids to LPS or probably lipid A.

In previous papers (1, 2), we reported that the difference of febrile response among rabbits, rats and chickens to endotoxin (LPS) depended grossly on inactivation of the pyrogenicity by cerebral proteolipids derived from these three species.

In the present paper, we further studied the mechanism of inactivation of pyrogen by three cerebral proteolipids in vitro, especially the binding profiles of LPS and these cerebral proteolipids isolated by Sephadex LH-20 column chromatography. In addition, the binding capacities to LPS of these three proteolipids and some phospholipids were compared.

MATERIALS AND METHODS

Animals: Male domestic rabbits weighing 2.0–2.8 kg, male Wistar rats weighing 150–200 g, and male adult chickens weighing 1.5–2.0 kg were used for pyrogen tests and/or for preparation of cerebral proteolipids by the method of Folch and Lees (3). Proteolipids were purified by ether precipitation according to Soto et al. (4).

Column chromatography: Sephadex LH-20 (Pharmacia, Uppsala) in 2.5×26 cm columns was equilibrated with chloroform
proteolipids applied to the column, and chemical analyses of the chemical components in each fraction (8 ml/tube) were done according to the previous paper (2).

Preparation of bacterial pyrogen: LPS was extracted from cultured E. coli UKT-B strain cells by the hot-phenol method (5) and further purified by ultracentrifugation (105,000×g, 4 hr×2). Before incubation with proteolipids, lyophilized LPS was made into a gel by admixing with a little distilled water and then mixing by a Vortex mixer with the Folch system lower phase solvent (CHCl₃-CH₃OH-H₂O; 86:14:1). A transparent solution was obtained by these procedures.

Chemicals and chemical analyses: Lecithin, lysolecithin, L-α-phosphatidylcholine diaroyl, L-α-phosphatidylcholine dimyristoyl, L-α-phosphatidylcholine dioleoyl, L-α-phosphatidylcholine distearoyl, L-α-phosphatidylcholine dipalmitoyl and sodium dodecyl sulfate (SDS) were purchased from the Sigma Chemical Co. Protein was determined by the method of Hess and Lewin (6), total lipid phosphorus according to the method of Volkin and Cohn (7) and galactose by the orcinol-sulfuric acid reaction (8).

Binding assay of proteolipids to LPS: The binding capacity of proteolipids to LPS was investigated by the biphasic solvent system (9). LPS was determined by the anthrone-sulfuric acid reaction (10). The proteolipid solution was exhaustively washed with distilled water until the aqueous phase became negative in the anthrone-sulfuric acid reaction. After stirring the equal volumes of LPS solution in water and the proteolipid solution in a C/M mixture (2:1) at room temperature for 15 min, the bound LPS was calculated by determining the quantity of LPS remaining in the upper phase.

Others: Other materials and methods are described in "RESULTS".

RESULTS

Comparison of the elution profiles of the mixtures of LPS and proteolipids derived from rabbit, rat and chicken cerebrums on Sephadex LH-20: The chromatographic elution profiles of the mixtures of LPS and proteolipids extracted from cerebrums of three species are shown with controls in Fig. 1. Protein of rabbit proteolipid was eluted in two separate effluents of C/M (6:1 and 4:1), whereas those of rat and chicken proteolipids were mainly in the C/M (4:1) effluent. Significant differences in the elution profiles including lipid phosphorus and cerebrosides estimated as galactose could not be detected among the three sources of proteolipid.

After incubation of the above three proteolipids (5 mg as protein) with LPS (1 mg) at 37°C for 30 min, the incubation mixtures were applied to the column, and the elution profiles of chemical components were checked. The elution profiles of lipid phosphorus and galactose of both rat and chicken proteolipids were unchanged by incubation with LPS. However, a new protein peak appeared in the chloroform effluent chromatogram of the incubation mixtures of these cerebral proteolipids with LPS.

Pyrogenicity of the new protein peak which appeared in chloroform effluent chromatographs of the incubation mixtures of LPS and proteolipids: The pyrogenicity of each fraction of the new protein peak (in the chloroform effluent shown in Fig. 1. (B)) was determined by the same method described in the previous report (2). The results are summarized in Table 1. By chromatography of LPS (1 mg) alone, pyrogenicity was not detected in the chloroform effluent, but was detected in the more polar effluents of C/M (6:1 and 4:1). The chloroform effluent fractions of the new protein peak were not pyrogenic except for
Fig. 1. Comparison of the elution profiles of cerebral proteolipids derived from certain species on a Sephadex LH-20 column. Top: rabbit, middle: rat, bottom: chicken. Column size: 2.5×26 cm, fraction volume: 8 ml/tube, flow rate: 0.5 ml/min.

No. 6, but those fractions became markedly pyrogenic upon treatment with 2% SDS. These results are in line with our previous findings on rabbit proteolipid (2).

Inactivation effect of various phospholipids on the pyrogenicity of LPS: In a previous report (2), it was demonstrated that the phospholipid fractions of the chloroform effluent (Fr. I) could also inactivate LPS pyrogenicity. Therefore, the pyrogenic inactivating potencies of some phospholipids including lecithin, lysolecithin, L-α-phosphatidylcholine distearoyl and L-α-phosphatidylcholine dioleoyl were compared with that of rabbit cerebral proteolipid. As shown in Fig. 2, the pyrogenic inactivating potencies of lysolecithin, lecithin and L-α-phosphatidylcholine distearoyl were less active. L-α-phosphatidylcholine dioleoyl, however, showed a potent inactivating effect, but the
Table 1. Distribution of pyrogenicity in the chloroform eluate and its change by treatment with 2% SDS after chromatography of LPS or the mixture of LPS plus rat or chicken proteolipid (PL) on Sephadex LH-20

| Fraction Number | LPS alone  | 2% SDS(+) | 2% SDS(-) | LPS plus Rat PL | 2% SDS(+) | 2% SDS(-) | LPS plus Chicken PL | 2% SDS(+) | 2% SDS(-) |
|----------------|-----------|-----------|-----------|----------------|-----------|-----------|---------------------|-----------|-----------|
| 2              | <0.6      | <0.6      | <0.6      | <0.6          | <0.6      | <0.6      |
| 4              | <0.6      | <0.6      | <0.6      | <0.6          | <0.6      | <0.6      |
| 6              | <0.6      | <0.6      | 1.5       | 1.8           | 1.0       | 2.4       |
| 8              | <0.6      | <0.6      | <0.6      | 0.7           | <0.6      | 1.3       |
| 10             | <0.6      | <0.6      | <0.6      | <0.6          | <0.6      | 1.1       |
| 12             | <0.6      | <0.6      | <0.6      | <0.6          | <0.6      | 1.3       |
| 14             | <0.6      | <0.6      | <0.6      | <0.6          | <0.6      | 1.0       |
| 16             | <0.6      | <0.6      | <0.6      | <0.6          | <0.6      | <0.8      |

Results are expressed as maximum increase in rectal temperature (JT°C). Conditions of the chromatography are the same as Fig. 1. One tenth ml of the eluate of each fraction (8 ml) was dried under N2 and dissolved in 10 ml of saline before injection into rabbits. SDS treatment was carried by mixing 0.1 ml of the eluate with 0.1 ml of 2% SDS under N2 before dilution with saline.

Fig. 2. Inactivation of LPS pyrogenicity by rabbit cerebral proteolipid or some phospholipids. One tenth ml of LPS solution (100 µg/ml) was incubated with 0.9 ml of proteolipid or phospholipids solution at 37°C for 30 min and then diluted 1,000-fold with saline before injection. Dose of LPS=0.01 µg/kg i.v. The fever index for 4 hours (F.I.-4) was calculated from the curves of the mean value (JT°C) in 3 rabbits according to the method described in a previous report (1). -○-: proteolipid, -X-: L-α-phosphatidylcholine dioleoyl, -●-: L-α-phosphatidylcholine distearoyl, -△-: lecithin, -▽-: lysolecithin.

inactivating effect was not a linear function of the phospholipid concentrations.

Binding capacity to LPS of proteolipids derived from cerebrrums of the three species and various synthetic lecithins: By the biphasic solvent system (9), the binding capacities of the three sources of proteolipid and the various synthetic phospholipids were determined. As shown in Fig. 3, the order of the binding capacity of the proteolipids derived from the three species was as follows; chicken, rat and rabbit. These results are in parallel to the pyrogenic inactivating potency previously described (1).

On the other hand, the synthetic lecithins (L-α-phosphatidylcholine dilauroyl, L-α-phosphatidylcholine dimyristoyl, L-α-phosphatidylcholine dioleoyl, L-α-phosphatidylcholine dipalmitoyl) could not bind to LPS even at high concentration (10 mg/ml).

DISCUSSION

We compared in this report the elution profiles of proteolipids derived from rabbit, rat and chicken on a Sephadex LH-20
Fig. 3. Binding capacity of cerebral proteolipids derived from certain species and synthetic lecithins to LPS. After stirring the equal volumes (1.5 ml) of LPS solution (0.2 mg/ml) in water and proteolipid or lecithin solution (0.01-10 mg/ml) in the C/M mixture (2:1) at room temperature for 15 min, the bound LPS was calculated by determining the quantity of LPS remaining in the upper phase. —O—: chicken proteolipid, ——: rat proteolipid, —-—: rabbit proteolipid, —-—: synthetic lecithins (L-α-phosphatidylcholine-dilauroyl, -dimyristoyl, -dioleoyl, -dipalmitoyl) column. There was no significant difference in the elution profiles of both lipid phosphorus and cerebrosides among the three proteolipids, but the protein of rat and chicken cerebral proteolipids was eluted mainly in the C/M (4:1) effluent, whereas that of rabbit cerebral proteolipid was eluted mainly in the effluents of both C/M (6:1) and (4:1) (Fig. 1, (A)). On chromatographing the incubation mixtures of the cerebral proteolipids of the three species with LPS, a new protein peak appeared in the chloroform effluent in all cases (Fig. 1, (B)). Fractions of these protein peaks were markedly pyrogenic after treatment with 2% SDS (Table 1), thereby indicating that the de novo formed complex of proteolipid-protein with LPS was dissociated by SDS. The results were similar to that with rabbit proteolipid described in our previous paper (2).

Inactivation curves of LPS pyrogenicity by rabbit cerebral proteolipid and the related phospholipids indicated that both cerebral proteolipid and L-α-phosphatidylcholine dioleoyl clearly inactivated LPS pyrogenicity. However, their mechanisms of inactivation may be different from each other; the former could directly bind to LPS, but the latter might make active centers of LPS (lipid A) aggregates based on a nonpolar radical of the lipid and not bind to LPS. This was further confirmed by measuring the binding capacity to LPS of cerebral proteolipid and various synthetic phospholipids (Fig. 3). It was found that the binding capacity of the protein of cerebral proteolipid to LPS was in the following increasing order; chicken, rat, and rabbit. This meant that pyrogenic inactivation grossly paralleled the binding capacity of proteolipid-protein, and the pyrogenic inactivation by phospholipids did not include the binding process but another mechanism, micell formation with LPS (11).

At present, however, it is still uncertain whether the difference in interaction of the proteolipids with LPS among the three species depends on differences in physicochemical properties of these proteolipid-proteins.

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