INTERACTIONS BETWEEN BACTERIAL PYROGEN AND PROTEOLIPID EXTRACTED FROM THE CEREBRUM (II)

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Abstract—Our previous finding that the cerebral proteolipid could inactivate the pyrogenicity of lipopolysaccharide (LPS) in vitro was also studied by Sephadex LH-20 column chromatography and the following results were obtained. When rabbit cerebral proteolipid was chromatographed, two main protein peaks were obtained. One appeared in the chloroform (C)/methanol (M) 6:1 and the other C/M 4:1 effluent, designated as fraction IV and fraction V, respectively. When the incubation mixture of proteolipid and LPS was chromatographed, a new protein peak appeared in the C effluent. The new protein peak was suggested to be a complex of proteolipid protein and LPS, because pyrogenicity could be detected in the protein fractions only after treatment with 2% SDS. Fraction V but not fraction IV inactivated the pyrogenicity of LPS in vitro. By re-chromatography of the incubation mixture of fraction V and LPS, a complex of protein and LPS was also eluted in the C effluent. On the other hand, by re-chromatography of the incubation mixture of fraction IV and LPS, such a complex was not detected in the C effluent. The present results suggest that the proteolipid apoprotein eluted in the C/M 4:1 effluent on a Sephadex LH-20 column plays an important role in the inactivation of the pyrogenicity of LPS.

In a previous paper (1), we reported that the species specificity of pyrogenic response was observed among rabbits, rats and chickens by i.v. administration of bacterial endotoxin (lipopolysaccharide, LPS) and was related to the in vitro inactivation effect of each cerebral proteolipid on the pyrogenicity of LPS. These data suggested that LPS might interact with proteolipid during the penetrating process into central nervous system and that the pyrogenicity of LPS might be modified by proteolipid. In the present study, we investigated the in vitro interaction between LPS and proteolipid of rabbit cerebrum by Sephadex LH-20 column chromatography.

MATERIALS AND METHODS

Experimental animal: Male Japanese domestic white rabbits weighing 2.0–2.8 kg were used for pyrogen test and preparation of cerebral proteolipid.

Preparation of LPS and pyrogen test: Preparation of LPS from E. coli UKT-B strain was carried out according to the method of phenol-water extraction (2). The pyrogenicity of the samples was determined by i.v. administration to three rabbits and expressed as the mean of the maximum increase in body temperature for five hours.

Preparation of proteolipid: Rabbit cerebral proteolipid was extracted from white and gray
matter of cerebrum by the method of Folch et al. (3) and purified by the ether precipitating method of Soto et al. (4).

Column chromatography: Columns measuring 2.5×26 cm were used in all cases. Sephadex LH-20 (Pharmacia, Uppsala) was equilibrated with chloroform. Aliquots of 8 ml/tube was collected with a fraction collector at 0.5 ml/min at room temperature. Elution was carried out stepwise with chloroform (C)-methanol (M) mixture of increasing polarity. In the chromatography of the intact proteolipid, the effluent was separated into the following five fractions; fraction I (C alone), fraction II (C/M 19:1), fraction III (C/M 15:1), fraction IV (C/M 6:1) and fraction V (C/M 4:1).

Chemical analysis: Protein was determined by the method of Hess and Lewis (5), total lipid phosphorus according to Volkin and Cohn (6) and galactose by the orcinol-sulfuric acid reaction (7).

RESULTS

Elution pattern of proteolipid on a Sephadex LH-20 column: The chromatographic pattern of the proteolipid on a Sephadex LH-20 column is represented in Fig. 1. Two main protein peaks were observed in the effluent of C/M (6:1) and C/M (4:1). Lipid phosphorus was eluted with chloroform alone and most cerebrosides estimated as galactose were eluted in the effluent of C/M mixture of both 19:1 and 6:1. The reproducibility of the elution patterns was confirmed in several experiments and the re-elution patterns were not influenced by temperatures used for the incubation of proteolipid.

Elution pattern of proteolipid incubated with LPS on a Sephadex LH-20 column: After incubation of proteolipid (20 mg as protein) with LYS (1 mg) at 37°C for 30 min in vitro, the incubation mixture was chromato-
graphed on the column and each fraction was admitted to the quantitative analysis of phosphorus, galactose and protein. As shown in Fig. 2, a new peak of protein appeared in the chloroform effluent between fraction No. 9 and No. 16, but patterns of phosphorus and galactose were similar to those of Fig. 1.

**Pyrogenicity of each fraction derived from the chromatography of LPS and LPS plus proteolipid:** It was impossible to show the elution pattern of LPS from chromatography of LPS alone or the incubation mixture of proteolipid and LPS by chemical analysis, because only a small amount of LPS (1 mg) was applied on the column. Therefore, the pyrogenicity of each fraction was estimated before and/or after addition of 2% SDS. As shown in the summarized data of Table 1, the pyrogenicity in the chromatography of LPS alone was observed in the effluent of C/M (6:1) and C/M (4:1), but in the incubation mixture of proteolipid and LPS, it was observed only in the effluent of C/M (6:1). After treatment with 2% SDS, however, the pyrogenicity was clearly demonstrated in the effluent of chloroform in addition to both the C/M (6:1) and the C/M (4:1) effluent.

**Inactivation effect of each fraction derived from proteolipid on the pyrogenicity of LPS:**
The five fractions derived from the parent proteolipid shown in Fig. 1 were condensed to 1/10 volume in vacuo, and we assessed which fractions could inactivate the pyrogenic activity of LPS in vitro. The above condensed fractions were incubated with LPS at 37°C for 30 min, and the incubation mixtures were diluted with saline before given i.v. to rabbits. The data are shown in Fig. 3. It was observed that the fever response induced by the five incubation mixtures could be significantly divided into the two groups as follows; inactivation of the LPS pyrogenicity was observed in fraction I (C alone) and V (C/M 4:1), while the other fractions were all negative.

Inactivation effect of fraction IV and V on the pyrogenicity of LPS: From the experiments shown in Table 1 and Fig. 3, it was suggested that fraction V (C/M 4:1) could bind to LPS thereby inactivating the pyrogenicity, but fraction IV (C/M 6:1) could

| Solvent Ratio | Tube Number | LPS alone | LPS plus proteolipid |
|---------------|-------------|-----------|----------------------|
|               |             | 2% SDS(−) | 2% SDS(+)           |
| (1−20)        | JT°C        | JT°C      | JT°C                 |
| 2             | <0.6        | <0.6      | <0.6                 |
| 4             | <0.6        | <0.6      | <0.6                 |
| 6             | <0.6        | 0.8       | 1.8                  |
| 8             | <0.6        | <0.6      | 1.7                  |
| C alone       | 10          | <0.6      | <0.6                 |
|               | 12          | <0.6      | 1.2                  |
|               | 14          | <0.6      | <0.6                 |
|               | 16          | <0.6      | <0.6                 |
|               | 18          | <0.6      | <0.6                 |
|               | 20          | <0.6      | <0.6                 |
| C/M, 19:1     | 21−30       | <0.6      | <0.6                 |
| C/M, 15:1     | 31−40       | <0.6      | <0.6                 |
| (41−50)       |             |           |                      |
| 42            | <0.6        | <0.6      | <0.6                 |
| 44            | <0.6        | 1.3       | 1.4                  |
| C/M, 6:1      | 46          | 1.5       | 1.2                  |
|               | 48          | 1.5       | 0.8                  |
|               | 50          | 1.3       | 1.6                  |
| (51−60)       |             |           |                      |
| 52            | 2.7         | <0.6      | 0.8                  |
| 54            | 1.5         | <0.6      | 1.6                  |
| C/M, 4:1      | 56          | 1.7       | <0.6                 |
|               | 58          | <0.6      | <0.8                 |
|               | 60          | <0.6      | <0.6                 |

Conditions of the chromatography are the same as Fig. 1. One-tenth ml of the eluate of each tube (8 ml) was dried under N₂ and dissolved in 10 ml of saline before injection into rabbits. SDS treatment was carried out as follows: 0.1 ml of the eluate was mixed with 0.1 ml of 2% SDS under N₂ before dilution with saline. C = chloroform, M = methanol.
not interact with LPS. To further clarify the results, the two fractions were re-chromatographed on the column after incubation with LPS in vitro at 37°C for 30 min. The pyrogenicity of each fraction tube was assayed as usual by injection into rabbits before and after treatment with 2% SDS. The protein content of each fraction tube was also determined.

By the re-chromatography of the incubation mixture of fraction V (C/M 4:1) and LPS, a new protein peak appeared in the chloroform effluent, that corresponding to a significant decrease in protein content of the effluent of C/M (4:1). On the contrary, with re-chromatography of the incubation mixture of fraction IV (C/M 6:1) and LPS, the elution pattern of protein was not changed by LPS, i.e. only a single peak of protein was observed in the effluent of C/M (6:1) (Data not shown).

As shown in Table 2, when the incubation mixture of fraction IV (C/M 6:1) and LPS was re-chromatographed, a new protein peak appeared in the chloroform effluent, corresponding to a significant decrease in protein content of the effluent of C/M (6:1). On the contrary, with re-chromatography of the incubation mixture of fraction IV (C/M 6:1) and LPS, the elution pattern of protein was not changed by LPS, i.e. only a single peak of protein was observed in the effluent of C/M (6:1) (Data not shown).

**Table 2. Distribution of pyrogenicity in the eluate and its change by treatment with 2% SDS after chromatography of the mixture of LPS plus Fr. IV or Fr. V on Sephadex LH-20**

| Solvent Ratio | Fr. IV plus LPS | Fr. V plus LPS |
|---------------|-----------------|----------------|
|               | Tube Number     | 2% SDS(-)     | 2% SDS(+)     | Tube Number     | 2% SDS(-)     | 2% SDS(+)     |
| (1-20)        | 2°C             | <0.6          | <0.6          | (1-20)          | 2°C             | <0.6          | <0.6          |
| 2             | <0.6            | <0.6          | 6             | <0.6            | <0.6          |
| 6             | <0.6            | <0.6          | 9             | <0.6            | <0.6          |
| 8             | <0.6            | <0.6          | 10            | <0.6            | 1.1          |
| 10            | <0.6            | <0.6          | 11            | <0.6            | 1.7          |
| C alone       |                 |               |               |                 | 12            | <0.6          | 1.4          |
| 12            | <0.6            | <0.6          | 12            | <0.6            | 1.4          |
| 14            | <0.6            | <0.6          | 13            | <0.6            | 1.0          |
| 18            | <0.6            | <0.6          | 14            | <0.6            | 1.5          |
| 20            | <0.6            | <0.6          | 15            | <0.6            | 1.7          |
|               |                 |               |               |                 | 16            | <0.6          | 1.9          |
|               |                 |               |               |                 | 17            | <0.6          | 1.3          |
| (31-45)       |                 | 0.8           | 25            | 1.5             | 1.1          |
| 32            | <0.6            | 0.8           | 25            | 1.5             | 1.1          |
| 34            | 1.2             | 1.7           | 28            | 2.5             | 2.3          |
| 36            | 1.7             | 1.9           | 28            | 2.5             | 2.3          |
| C/M, 6:1      |                 |               |               |                 | 1.3          |
| 38            | 2.6             | 2.3           | 34            | 1.1             | 3.3          |
| 40            | 2.3             | 2.4           | 36            | 1.0             | 2.6          |
| 42            | 1.5             | 1.8           | 38            | 1.0             | 3.3          |
| 44            | 2.1             | 1.6           | 45            | 1.5             | 1.6          |
| (46-55)       |                 |               |               |                 | 50            | 1.3           | 1.3          |
| 47            | 1.3             | 2.3           | 34            | 1.1             | 3.3          |
| 51            | 1.5             | 1.0           | 36            | 1.0             | 2.6          |
| 53            | 1.1             | 1.0           | 38            | 1.0             | 3.3          |

Fr. IV (C/M: 6:1 protein fraction) and Fr. V (C/M: 4:1 protein fraction) shown in Fig. 1 were pooled respectively, and condensed to 1/10 volume in vacuo before incubation with LPS. Conditions of the chromatography and the treatment of the eluate are the same as Table 1. C=chloroform, M=methanol.
mixture of fraction IV (C/M 6:1) and LPS was re-chromatographed, the pyrogenicity could be detected only in the effluent of both C/M (6:1) and C/M (4:1), similar to the results seen with LPS alone described in Table 1. On the other hand, by treatment with SDS, the pyrogenicity could be clearly detected in the chloroform effluent (the new protein peak) which was obtained by the re-chromatography of the incubation mixture of fraction V (C/M 4:1) and LPS. From these data, it should be stressed that only fraction V (C/M 4:1) could bind to LPS and inactivate its pyrogenicity in vitro.

**DISCUSSION**

In a previous report (1), we suggested that the species specificity of LPS-induced pyrogenic response in vivo might be related to the in vitro inactivation effect of each cerebral proteolipid on the pyrogenicity of LPS. In the present experiments, we found that the elution profile of proteolipid pre-incubated with LPS was markedly different from that of proteolipid alone on a Sephadex LH-20 column, that is, a new protein peak appeared in the chloroform effluent (Fig. 2). The new protein peak was suggested to be a complex of proteolipid protein and LPS (Table 1). It has been generally accepted that LPS was constructed with lipid A as a pyrogenic principle and hydrophilic polysaccharide (8). Therefore, it is likely that lipid A moiety of LPS is efficiently bound to a site of lipophilic proteolipid protein, particularly fraction V eluted with C/M (4:1) on the column and that the de novo formed complex of LPS and proteolipid protein is dissociated by SDS treatment (Table 2).

Inactivation effect of fraction V was clearly demonstrated in Fig. 3. In addition to fraction V, however, Fig. 3 shows that fraction I in which most of phospholipids were eluted could also inactivate the pyrogenicity of LPS. At present it is obscure whether lipid moieties of proteolipid interact with LPS as the protein of fraction V, since the lipid elution patterns was not altered by incubation of proteolipid with LPS (Fig. 2). As for the interaction of phospholipids and LPS, Springer and Adye (9) reported that phospholipids extracted from human leucocytes and platelets by butanol could inhibit coating of erythrocytes by LPS and suggested that LPS-binding activity of leucocytes and platelets was confined to their lipid moiety and predominantly to the phospholipids. However, it is uncertain that phospholipids bind to LPS, since the LPS-binding activity was indirectly evidenced by the ability of phospholipids to inhibit the attachment of LPS to erythrocytes. Shands (10) observed electron microscopically that a large micellar conformation of intact LPS was disaggregated into small pieces by the in vitro incubation with phospholipids, and stated that on attachment of a particle of LPS to a mammalian membrane, phospholipids interacting with the LPS particle might solubilize LPS and, by phase transitions similar to those of vesiculation, bring LPS into the membrane structure. Therefore, it is likely that the interaction between phospholipids and LPS is a certain micellar organization and that the inactivation of LPS pyrogenicity by fraction I as shown in Fig. 3 is nonspecifically induced as a result of a micellar organization. Purification of fractions I and V is underway.

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