International Immunopharmacology 9 (2009) 1518–1524

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Pharmacological characterization of anaphylaxis-like shock responses induced in mice by mannan and lipopolysaccharide

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ARTICLE INFO

Article history:
Received 23 June 2009
Received in revised form 27 August 2009
Accepted 4 September 2009

Keywords:
Shock
Mannan
Lipopolysaccharide
Platelet
Complements
Platelet-activating factor

ABSTRACT

Intravenous injection of lipopolysaccharide (LPS, a component of the Gram-negative bacterial cell-surface) or mannan (Man, a component of the fungal cell-surface) into mice reportedly induces anaphylaxis-like shock (ALS) via complement-associated platelet degradation and platelet-activating factor (PAF), respectively. However, it is unclear whether PAF is involved in LPS-ALS or whether complements and/or platelets are involved in Man-ALS. Here, using preparations of Man from *Saccharomyces cerevisiae* and LPS from *Klebsiella* O3, we characterized and compared LPS-ALS and Man-ALS, with the following results. (1) ALS depended on mouse strain (ddY and BALB/c being highly responsive to Man and LPS, respectively), but not on Toll-like receptors 2 and 4. (2) In ddY mice, Man had little effect on platelets, K76 (C5a-inhibitor) did not prevent Man-ALS, and Man-ALS was augmented by prior platelet depletion. (3) CV-3988 (PAF antagonist) prevented Man-ALS, but not LPS-ALS. (4) LPS-ALS and Man-ALS were each augmented by prior injection of a muramyl dipeptide (MDP, a constituent abundant in the Gram-positive bacterial cell-surface), but prevented by prior macrophage depletion. (5) Co-administration of Man and LPS induced an augmented ALS in both ddY and BALB/c mice. These results indicate that (i) Man and LPS each induces ALS in mice in strain-dependent and macrophage-dependent (but not TLR-dependent) ways by stimulating a platelet-non-associated PAF pathway and a platelet-associated complement pathway, respectively, and (ii) these pathways are primed by MDP and exhibit mutually augmenting actions. Man-ALS and LPS-ALS may therefore serve as models for diseases involving augmentation by multiple or mixed infections.

1. Introduction

Intravenous injection into mice of lipopolysaccharide (LPS, a cell-wall component in Gram-negative bacteria) is reportedly followed "within several minutes" by anaphylaxis-like shock (ALS), and the induction of the ALS is dependent both on the structure of the LPS and on the strain of mice [1,2]. This LPS-ALS is thus different from the so-called "endotoxin shock", which becomes evident in mice several hours after an injection of LPS. Endotoxin shock is induced via stimulation of Toll-like receptor 4 (TLR4), and so C3H/HeJ mice, which have mutated TLR4, are resistant to endotoxin shock [3,4]. Muramyl dipeptide (MDP, a cell-wall component abundant in Gram-positive bacteria) is an immunologically active unit of peptidoglycan, and MDP is recognized by a nucleotide-binding oligomerization domain (NOD)-like receptor-2 (NOD2) [5,6]. Pretreatment with MDP augments both LPS-ALS and endotoxin shock in mice, including C3H/HeJ mice [1,2,7]. Injection of whole cells of streptococci (Gram-positive bacteria) also induces ALS [8]. Interestingly, the induction of both LPS-ALS and *Streptococcus*-ALS involves activation of platelets followed by their degradation in the lung, liver, and/or circulation [9,10], and this platelet response is induced by activation of the lectin pathway in the complement system [8,11,12]. In the *Klebsiella* O3 LPS molecule, the mannose homopolymer structure within the O-antigen region is important for stimulation of the lectin pathway [12]. Thus, the ALS induced in mice via complement-associated platelet degradation is a typical disease model for the rapid innate immune responses that occur upon bacterial infection, irrespective of whether the bacteria concerned are Gram-positive or Gram-negative.

Fungal species not only cause mycoses, including endocarditis [13], but also modulate the immune system [14–18]. Mannan (Man) is the major cell-surface component of fungi, and it has been supposed that Man is recognized by the host's innate immune system [14,17,19]. Activation of the coagulation pathway (including platelets), with the development of a fibrin clot, is an early event in the development of

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doi:10.1016/j.intimp.2009.09.006

many thrombotic diseases, including endocarditis [20,21]. Interestingly, like LPS, extracts from yeast cells or preparations of Man induce ALS when injected intravenously into mice [22–27]. Moreover, it is reported that platelet-activating factor (PAF) is causally involved in Man-ALS [26,27]. Although it is not known whether PAF is involved in LPS-ALS or whether complements and/or platelets are involved in Man-ALS, PAF is known to be a potent stimulator of platelets, leading to their aggregation, suggesting that platelets may indeed contribute to Man-ALS. Strangely, however, murine platelets are reported to be unresponsive to PAF [28,29]. In addition to its effect on platelets, PAF plays important roles in the activation of immunocompetent cells such as neutrophils, macrophages, and endothelial cells [30,31]. This suggests that like the complement system, the PAF system (including the synthesis and degradation of PAF, and the expression of its receptors) may be involved in innate immunity. Like those complement components called "anaphylatoxins" (C3a, C4a, and C5a), PAF is a typical mediator of ALS [30,31]. Interestingly, increasing evidence suggests that PAF and platelets are involved even in acquired immunity (i.e., in allergies, including anaphylaxis and asthma) [31,32].

The importance of innate immunity lies in the rapidity with which it permits the host to respond to invaders. Having considered the background described above, we became interested in such rapid reactions as the ALS responses induced by injection of various microbial substances because we thought that their analyses might inform our understanding of the rapid nature of innate immunity. In the present study, using *Saccharomyces cerevisiae* Man and *Klebsiella* O3 LPS, we characterized and compared Man-ALS and LPS-ALS.

2. Materials and methods

2.1. Animals

Male ddY, C57BL/6, C3H/HeN, C3H/HeJ, AKR, and DBA/2 mice (5–6 weeks old) were purchased from SLC Japan (Shizuoka, Japan). Male BALB/c mice (6 weeks old) were provided by the animal facility of our university, and were raised in our laboratory. TLR2-knockout (KO) mice (C57BL/6 background) were provided by Prof. Akira Shizuo [33]. Animal experiments were carried out at a room temperature of 27–28 °C, because LPS-induced ALS is reproducibly induced at this room temperature [12]. All procedures complied with the Guidelines for the Care and Use of Laboratory Animals in Tohoku University.

2.2. Materials

The neutral Man was purified from crude Man obtained from bakers' yeast, which was a wild-type strain of *S. cerevisiae* (Oriental Yeast Industries, Tokyo), as described previously [26]. The possible endotoxin contamination in the Man preparation was assessed in terms of *Limulus* activity using Endospecy ES-50M (Seikagaku Biobusiness Co., Tokyo) as described in [34]. The *Limulus* activity of the preparation was found to be 8.08 Endotoxin Unit (EU)/mg, which was estimated to be equivalent to 0.89 ng/mg of the reference LPS prepared from *Escherichia coli* 113:H10 (9.09 EU/ng). LPS from the *Klebsiella* O3 strain LEN-1 (S type) was prepared by the phenol–water method [35]. The MDP employed here (*N*-acetylmuramyl-L-alanyl-D-isoglutamine) was purchased from the Protein Foundation Peptide Institute (Osaka, Japan). A complement C5 inhibitor, K76 [36], was provided by Ohtsuka Pharmaceutical Co. (Tokushima, Japan). PAF and a PAF antagonist, CV-3988, were purchased from Sigma (St Louis, MO, USA). A rat monoclonal anti-mouse platelet antibody, Pm1, was kindly provided by Dr. T. Nagasawa of the University of Tsukuba (Tsukuba, Japan) [37]. Although the molecular antigen for Pm1 has not been identified, Pm1 has been shown to deplete platelets in mice [38]. Control IgG was prepared from normal rat serum by precipitation with ammonium sulfate, followed by dialysis of the precipitant. All the above reagents were dissolved in sterile saline, and they were injected intraperitoneally (i.p.), intravenously (i.v.) via a tail vein (0.1 ml/10 g body weight), or subcutaneously (s.c.) in the upper back. K76 was dissolved in saline with the addition of enough NaOH solution to bring the pH to 7.5. Experimental protocols are described either in the text or in the legend to the figure or table relating to each experiment.

2.3. Platelet counting and estimation of platelet translocation into the liver and lung

The required procedures were carried out as previously described [12,32,39]. Two or three drops of blood from each decapitated mouse were directly collected into a test tube for the measurement of platelet count or 5HT. Briefly, to measure the platelet count, blood was collected into a pre-weighed test tube containing 1.0 ml of 4 mM EDTA in 0.01 M phosphate-buffered saline (pH 7.0). The tube plus blood was weighed, and thus the weight of the blood could be calculated. The number of platelets was then (within 30 min) ascertained using a cell counter (Sysmex SF-3000; Toa Medical Electronics Co. Ltd., Kobe, Japan) and expressed as platelet count/g of blood. To measure 5HT, blood was collected into a pre-weighed tube containing 3 ml of 0.4 M $HClO_4$, 0.1% *N*-acetylcysteine-HCl, and 4 mM EDTA-2Na. After reweighing, platelets were destroyed by sonication, and each tube was cooled in an ice bath. Determination of the 5HT level in the blood was carried out soon after the blood was collected. To measure 5HT in tissues, livers and lungs were rapidly removed after blood collection, kept in a jar containing dry ice until needed, and their 5HT levels determined within 2 days. After extraction, 5HT was separated by column chromatography and measured fluorometrically, as previously described [40]. The levels of 5HT in the blood and tissues are expressed as nmol/g of blood or tissues.

2.4. Scoring of rapid shock induced by Man, LPS, and PAF

The maximal severity of the rapid shock seen in each mouse within 1 h of an injection of Man or LPS was scored as described elsewhere [12], the scoring system being: 0 (no signs of shock), 1 (staggering), 2 (crawling and prostration), 3 (prostration and weak convulsions), 4 (prostration and strong convulsions), and 5 (death). The shock-scoring data are expressed as scores for individual mice or as mean ± SD, as appropriate. The score estimation was confirmed by a blinded assistant. For the rapid shock induced by injection of PAF, the scoring was performed as described above, except that it was done during the first 30 min after the injection (because PAF-induced shock signs were evident within this period, but not thereafter).

2.5. Depletion and detection of macrophages

Clodronate-encapsulated liposomes (Clo-lip) selectively deplete phagocytic macrophages [41]. As described in previous publications [42,43], a 5-fold-diluted suspension of Clo-lip was intravenously injected into mice, and depletion of macrophages was confirmed by immunostaining of livers using F4/80 antibody (Serotec, Kidlington, UK).

2.6. Statistical analysis

Experimental values for 5HT levels and platelet counts are given as mean ± SD. The statistical significance of differences was assessed using a Student's unpaired *t* test or a Bonferroni post-hoc test. The difference in shock scores between the two experimental groups was analyzed using a Ridit (relative to an identified distribution) test [44], a non-parametric test.