HOST RESPONSE TO INFECTION

Promotion of platelet aggregation by sera from brucellosis patients with antiphosphatidylcholine antibodies

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Results obtained in this study suggest that in human brucellosis there is an antibody response against platelet-activating factor (PAF) and phosphatidylcholine (PC). The specificity of the antiphospholipid response was determined by inhibition assays. The PAF molecule was able to inhibit the anti-PC activity of the brucellosis-control serum. This inhibition capacity of PAF was similar to that of the phosphorylcholine (PYC) group. These results suggest that the inhibition activity could be attributed to the PYC group present in both PAF and PC molecules. Consequently, these findings support an immunodominant role of PYC in the antiphospholipid response of brucellosis. Furthermore, sera from patients infected with *Brucella* organisms were able to cause platelet aggregation, as were brucella phospholipids, suggesting a possible role of the antiphospholipid antibodies and phospholipids in the inflammatory response in brucellosis.

Introduction

Platelet activating factor (PAF) is a biologically active phospholipid that has several functions as well as platelet activation, including activation of polymorphonuclear leucocytes (PMNLs) and other leucocytes, alteration of vascular permeability and blood pressure, and contraction stimulation, [1]. It acts under normal physiological conditions but also mediates some pathological responses, most significantly in inflammatory and allergic processes. Evidence of these functions is found in its detection in blood samples of patients with arthritis [1–4]. The activation mechanism is still not fully known; however, it has been demonstrated that PAF acts as an intracellular and intercellular messenger, activating the target cells [5, 6].

Structurally, PAF is an ether-phosphorylcholine (ether-PYC) (acetyl-glyceryl-phosphorylcholine) that contains a fatty acid linked to C₁ by an ether linkage, an acetyl residue in C₂ and a phosphorylcholine (PYC) residue in C₃ [7]. The acetyl group is implicated in the PAF

aggregating activity, although the PYC region is also critical for its biological activity [8].

Brucellosis is a systemic infection caused by gram-negative bacteria of the genus *Brucella*, which is characterised by the development of focal disease, inflammatory response and frequent arthritic processes [9]. One of the most peculiar characteristics of the membrane structure of *Brucella* spp. is the presence of phosphatidylcholine (PC) on its outer membrane [10], which is rare in most gram-negative bacteria. The structural relationship between PC and PAF [1], together with the presence of anti-PC antibodies in patients suffering from brucellosis [11], suggest that PC and anti-PC antibodies could play some role in those biological processes. Therefore, this study first investigated the presence of anti-PAF antibodies in sera from patients with brucellosis. Then, due to its structural similarity to PAF, brucella PC was investigated as an aggregating factor *in vitro*.

Materials and methods

Subjects and sera

Seven patients who were admitted to the Clínica Universitaria de Navarra (Pamplona, Spain) were included in the study. The diagnostic criteria were: (i) the isolation of *Brucella* spp. from blood or other body

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fluid or tissue specimens; and (ii) the presence of compatible clinical findings (fever, sweats, arthralgia, headache, weight loss, hepatomegaly, splenomegaly or signs of focal disease) together with the appearance of specific antibodies to *Brucella* at significant titres. Significant titres were considered to be a sero-agglutination test titre ≥ 160 , or a Coombs test titre ≥ 320 . After diagnosis, five serum samples were taken from each patient at various times: 15 days after initiating treatment, when treatment was completed and during the post-treatment follow-up, quarterly for a year.

The control sera used in the study were obtained by: (1) mixing sera from 10 patients diagnosed with brucellosis on the day of the onset of the disease (brucellosis-control); and (2) mixing sera from 10 healthy donors (negative control).

Reagents and brucella extract

PAF, egg PC and phosphorylcholine were obtained from Sigma-Aldrich Corporation (St Louis, MO, USA).

Brucella phospholipids were obtained from the rough strain *B. melitensis* 115 by the procedure described previously [10]. Briefly, flasks containing Trypticase Soy Broth (bioMérieux, Marcy l'Etoile, France) were inoculated with the strain and then incubated for 48 h at 37°C. After centrifugation at 7500 *g* for 15 min, 5 g of packed cells were dispersed in chloroform:methanol:water (4:5:10, v:v:v) and shaken for 20 min. Then, a fresh mixture of chloroform and water (1:1, v:v) was added, shaken for 30 min and filtered (0.8- μ m cellulose acetate membrane filters). The residue on the filter was added to 10 ml of chloroform, and dried under a nitrogen flow (dry residue of *brucella* phospholipids, PL-*brucella*).

ELISA-PC and ELISA-PAF

The ELISA was performed as described previously [11] with 5 μ g of the antigen (egg PC or PAF) dissolved in PBS (10 mM phosphate-buffered saline, pH 7.2) (50 μ g/ml) applied to ELISA microplates (Maxisorp, Nunc). After incubation at 37°C for 24 h, the solution was tipped off and the microplates were washed four times with PBS plus Tween-20 0.05% (PBST). Serum samples were first diluted in a separate test tube in PBST before addition to the ELISA plates. The plates were incubated at 37°C for 4 h. Two different anti-human immunoglobulin peroxidase conjugates were used: rabbit anti- α (Pierce) and γ (Nordic) chains, diluted 1 in 1000 in PBST. After incubation for 30 min at 37°C, the plates were washed and developed by incubation for 20 min at room temperature with 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid diammonium salt (Sigma) 0.01% in 0.1 M citrate buffer (pH 4.0) containing H₂O₂ 0.01%. The colorimetric values were determined by measuring the optical

density (OD) at 405 nm; samples with OD₄₀₅ ≥ 550 were considered positive [11].

Inhibition assays

Inhibition of antibody binding to PC was performed as described by Harris *et al.* [12] with some modifications. Serum samples (diluted 1 in 100 in PBS) were pre-incubated with PAF, egg PC or PYC at different concentrations (5, 1, 0.5 or 0.1 mg/ml of serum) for 4 h at 37°C, and then overnight at 4°C with constant mixing. The samples were then centrifuged at 21 000 *g* for 20 min to eliminate the immunocomplex. The inhibition assay with each substance was repeated twice. The new binding activities in 'ELISA-PC' (OD₄₀₅) were compared with those of the control sera. The results were reported as percentage inhibition, calculated as follows: percentage inhibition = [(OD₄₀₅ positive control sample inhibited – OD₄₀₅ negative control sample)/(OD₄₀₅ positive control sample – OD₄₀₅ negative control sample)] $\times$ 100.

In-vitro platelet aggregation assay

Platelets were obtained from 15 ml of human blood taken from healthy donors, at the time of the assay, into siliconised glass tubes containing trisodium citrate 3.8% (9:1, v:v) to avoid coagulation (Becton Dickinson, Franklin Lakes, NJ, USA). After centrifugation at 800 *g* for 10 min at 4°C, the upper portion was recovered, i.e., 'platelet-rich plasma' (PRP). A second centrifugation (2000 *g*, 5 min, 4°C) separated the 'platelet-poor plasma' (PPP) from the lower portion. PPP was added to PRP until a platelet concentration ranging from 2.5×10^5 to $3.5 \times 10^5/\mu$ l was obtained; 500 μ l was placed in each cuvette and 50 μ l of aggregating agent were added. The platelet aggregation process was monitored for 5 min with an aggregometer (Aggregometer II) that measures light transmission. Adenyl diphosphate (ADP; Menarini Diagnostics, Florence, Italy) 2 μ g/ml was used as a positive control for induction of platelet aggregation. The inhibitory capacity of the sera (brucellosis-control and the negative-control) on the PAF aggregating activity was studied by incubating 50 μ l of PAF (1 μ g/ml) with 50 μ l of the control serum (3 h, 37°C). 50 μ l of this solution were then added to each cuvette and the platelet aggregation was monitored for 5 min. The percentage aggregating activity of the solution was determined with respect to the aggregation induced by the same concentration of PAF. Similarly, the aggregating capacity of the same control sera on platelets (undiluted sera) was also studied.

Experiments were run in quintuplicate. The statistical significance of differences was determined after variance analysis by comparison of group means by Dunnett's procedure.

Results and discussion

The detection of anti-PC and anti-PAF antibodies was determined by ELISA as described previously [11]. The results indicated that an anti-PC response was accompanied by either IgA or IgG anti-PAF antibodies (Table 1). To confirm this parallel response, the evolution of the anti-PC and anti-PAF responses was compared in a group of patients with brucellosis. The results demonstrated that both responses followed a similar evolution with time (data not shown). Similarly, the presence of platelet-aggregating antiphospholipid (PL) antibodies in patients with systemic lupus erythematosus (SLE) and antiphospholipid syndrome has already been discussed and proposed by other authors [13–16].

The specificity of the anti-PL response was determined by inhibition assays, absorbing control sera (patients with brucellosis and healthy donors diluted 1 in 100 in PBS) with different substances. The inhibition of anti-PC activity in ELISA was higher when the brucellosis-control was pre-incubated with the PAF than with the PC molecule. The serum sample was absorbed twice with PC to reach the same level of inhibition as PAF (70%). Furthermore, the inhibition capacity of PAF was similar to that of the PYC group (PAF is an ether PYC) [7], suggesting that such activity could be attributed to its PYC group, as well as in PC (ether-PYC) [17]. Therefore, these results support an immunodominant role of PYC in the anti-PL and anti-PAF responses in brucellosis.

It has been demonstrated that PC can undergo a series

of transformations during the inflammatory progression. First, phospholipase A₂ associated with LDL or expressed by different cell types during inflammatory processes hydrolyses the fatty acid of the C₂ producing a smooth PC; secondly, when PC presents polyunsaturated fatty acids in the C₂ (this is the case with brucella PC) [17] it is oxidised, causing a short-chain PC. The molecules obtained during these processes (smooth PC and oxidised short chain PC) have similar structures to PAF. Consequently, it is possible that the PC of *Brucella* undergoes a series of transformations generating a structure similar to PAF. According to this, the antibody response detected against PAF may be the result of a specific anti-PAF response, and not just due to cross-reactions between PC and PAF.

Platelet aggregation assays were performed to ascertain the possible biological consequences of the presence of anti-PAF antibodies in patients with brucellosis. Contrary to what was initially expected, when the brucellosis-control serum was pre-incubated with PAF, instead of causing a reduction of the percentage aggregation by PAF neutralisation, it caused no appreciable change (100% versus 107%, respectively). In fact, the brucellosis-control serum caused platelet aggregation, while the negative-control serum did not have any effect (73% versus 3%, respectively; Table 2). Brucellosis-negative serum significantly reduced the PAF activity (67% versus 100%, $p < 0.05$). This indicates that some component in sera could block PAF aggregation, although there are no data to confirm this hypothesis. In this sense, it is possible that the aggregative activity detected with the brucellosis-positive serum (73%) could neutralise such a blocking

Table 1. Serological and clinical characteristics of the patients with brucellosis

Patient no.	ELISA-PC		ELISA-PAF		Evolution of disease (days)	Clinical manifestations
	IgG	IgA	IgG	IgA		
27	–	+	+	+	≤ 15	Myalgia, arthralgia
28	+	–	+	–	≤ 15	Arthralgia
29	–	–	–	–	≤ 15	Orchitis
30	+	+	+	+	≤ 15	Arthralgia
31	–	–	–	–	180	Arthralgia
33	+	–	+	–	96	Arthralgia
34	+	+	+	–	≤ 15	Arthralgia

Table 2. Platelet aggregation with the different agents assayed (5 min, 37°C)

Agent	Mean (SD) percent platelet aggregation*	Relative aggregation†
ADP (2 µg/ml)	85.4 (1.8)	141.7
Phospholipids <i>B. melitensis</i> (1 µg/ml)‡	50.0 (4.0)	83
PAF (1 µg/ml)	60.2 (1.8)	100
Pool sera from brucellosis patients (positive control)	43.6 (7.6)	73
Pool sera healthy donors (negative control)	1.8 (0.8)	3
PAF (1 µg/ml) + brucellosis-positive serum	63.6 (9.6)	107
PAF (1 µg/ml) + brucellosis-negative serum	40.2 (8.1)	66.7

*Mean (SD) of five experiments.

†Activity compared to aggregation by PAF 1 µg/ml.

‡Equivalent to PC 0.3 µg/ml.

effect (107%, PAF + brucellosis-positive serum *versus* 100%, PAF alone).

Furthermore, the structural similarity between PC and PAF mentioned above suggests that the PC of *Brucella* spp. could directly act analogously to PAF and have an agonistic activity after binding to PAF receptors. In fact, other phospholipids that activate PAF receptors have been described, although in general they are less potent than PAF itself, re-emphasising the importance of the residues in C₂ and C₁. Thus, Stremmler *et al.* [18] demonstrated that the oxidation of PC that contains a polyunsaturated fatty acid in C₂ (*Brucella*-PC has a polyunsaturated fatty acid in C₂, lactobacillic acid) produces a structure with high activity similar to PAF. Similarly, Smiley *et al.* [8] demonstrated that oxidised derivatives of PC activate human PMNLs *via* interaction with the PAF receptor. Therefore, it is possible that the action of phospholipase A₂ and the oxygen radicals, generated after the activation of phagocytic cells by *Brucella* transform brucella PC into molecules with a biological activity similar to PAF. Moreover, the results obtained in aggregation assays support this hypothesis, as brucella phospholipids caused 50% platelet aggregation at a concentration of 1 µg/ml (83% activity, in comparison to aggregation by PAF) (Table 2). In contrast, the egg PC did not show any activity.

Taken together, these results could explain why, on some occasions, patients with brucellosis show an altered pattern of coagulation, although this alteration has not been fully studied [19, 20].

A study with a bigger sample of patients is needed to confirm the above hypothesis. In any case, the following sequence of events could be suggested. After a brucella infection, a response mediated by anti-PL antibodies is generated, at least against PC with a PYC dominant group, a chemical group also present in the inflammatory molecule PAF. The inflammatory response also provokes the release of a series of mediators (including PAF), the expression of enzymes such as phospholipase A₂ and the release of toxic free oxygen radicals. As brucella PC presents a polyunsaturated fatty acid in C₂ it is susceptible to being hydrolysed by phospholipase A₂ and oxidised by free oxygen radicals, thus generating molecules with a PAF-type activity that would enhance the inflammatory response. Furthermore, the anti-PC antibodies would also contribute to the inflammatory response by producing platelet aggregation and the subsequent release of new inflammation mediators.

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