Brief report

Platelet-bound lipopolysaccharide enhances Fc receptor–mediated phagocytosis of IgG-opsonized platelets

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Platelets express Toll-like receptor 4 (TLR4), and this has been shown to be responsible for the thrombocytopenia induced by lipopolysaccharide (LPS) administration in vivo. We studied the role of LPS in mediating platelet phagocytosis by THP-1 cells in vitro by flow cytometry. Oposonization of platelets with an IgG monoclonal (W6/32) antibody or with IgG autoantibody-positive sera from patients with autoimmune thrombocytopenia (AITP) significantly enhanced platelet phagocytosis (P < .001). In contrast, platelet phagocytosis did not occur if platelets were bound with only LPS. If, however, the LPS-bound platelets were also opsonized with either W6/32 or autoantibody-positive sera with titers greater than 4, there was a significant and synergistic increase in Fc-dependent platelet phagocytosis (P < .001, P = .003, P = .048, and P = .047). These results suggest that, in the presence of antiplatelet antibodies, bacterial products can significantly alter platelet phagocytosis, and this may have relevance to how Gram-negative infections enhance platelet destruction in some patients with AITP. (Blood. 2007;109:4803-4805)

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Materials and methods

Antibodies

Sera prepared from the blood of 8 patients with AITP previously screened for platelet-associated autoantibodies were obtained from the laboratory of Dr John Freedman (Department of Laboratory Medicine, St Michael’s Hospital, Toronto, ON, Canada). Serum IgG autoantibodies were detected and titered by flow cytometry and then blinded (samples 1 to 8) for laboratory personnel performing the phagocytosis assays. The murine IgG2a anti–human major histocompatibility complex (MHC) class I monoclonal antibody (W6/32) was produced in vitro by hybridoma HB-95 (ATTC HB-95; American Type Culture Collection, Manassas, VA). F(ab’)2 fragments of W6/32 were produced as previously described18; purity by high-performance liquid chromatography (HPLC) analysis was more than 96%.

Platelets

Blood was obtained by venipuncture into trisodium citrate from healthy laboratory volunteers. This study had no direct contact with human patients. Approval was obtained from the St Michael’s Hospital Institutional Review Board. Platelet-rich plasma (PRP) was prepared, and platelets were counted and adjusted to 10^9/mL. Platelets were labeled with 20 µM CellTracker Green CMFDA (CM-G; Invitrogen, Eugene, OR), washed, and resuspended in PBS. Where indicated, CM-G–labeled platelets were either incubated with titrations of LPS and/or 5 µg W6/32 and/or a 1:2 dilution of...
human serum for 30 minutes at room temperature (RT) in the dark. Cell were washed once and used in the phagocytosis assay.

**Phagocytosis assay**

Human monocytic THP-1 cells (ATCC TIB-202) were counted, and $1 \times 10^7$ cells per milliliter were activated with 50 ng/mL phorbol 12-myristate 13-acetate for 15 minutes and washed. The phagocytic reaction was started by incubating $5 \times 10^6$ THP-1 cells with $250 \times 10^6$ platelets in 0.1 mL duplicate tubes for 60 minutes on ice or at 37°C. Extracellular fluorescence was then quenched by addition of 0.1% trypan blue. The mixture was centrifuged at 2000 g for 10 minutes at 4°C, the supernatant discarded, and 200 µL LDS DNA stain (FL3; Molecular Probes, Eugene, OR) added. Flow cytometry was performed using a FACSsort flow cytometer (Becton Dickinson, San Jose, CA), and acquisition was through a live electronic FL3 gate. Intracellular FL1 CM-G platelet fluorescence in the nucleated events was determined. The phagocytic index was calculated by the following formula: median FL1 fluorescence at 37°C/median FL1 fluorescence at 4°C. Statistical significance in panel A by a 1-way ANOVA was $P < .001$.

**Statistical analysis**

A 1-way analysis of variance (ANOVA) was used to compare the means of W6/32 phagocytosis, and a 2-tailed paired t test for comparison between means was used for the patient sera.

**Results and discussion**

We then attempted to reproduce the W6/32 results with antiplatelet autoantibodies from the sera of patients with AITP. By flow cytometry, 4 of 8 sera were negative for IgG antiplatelet autoantibodies, and 4 contained autoantibodies with titers ranging from 4 to 128. None of the autoantibody-negative sera could mediate THP-1 phagocytosis of platelets (Figure 2A, open bars), even when LPS was also bound to the platelets (Figure 2A, solid bars). In contrast, however, 3 of the 4 autoantibody-positive sera (those with the higher titers: 64, 64, and 128) opsonized human platelets and enhanced their phagocytosis by the THP-1 cells (Figure 2B, open bars). As with W6/32, when the autoantibody-opsonized platelets were also bound with LPS, their phagocytosis was significantly ($P = .031$, $P = .048$, $P = .047$) and synergistically enhanced (Figure 2B, solid bars) when compared with only opsonized platelets (Figure 2B, open bars). Thus, LPS in conjunction with IgG antiplatelet autoantibodies from patients with AITP can significantly enhance platelet phagocytosis.

These observations with LPS-bound platelets may be related to the recent reports demonstrating that platelets express TLRs and can bind LPS via TLR4.13-17 The mechanism of how platelet-bound LPS together with autoantibody opsonization synergizes to enhance platelet phagocytosis is unknown, but because the increase was Fc dependent (Figure 1C) it may suggest that the interaction of TLR- and FcR-mediated signaling...
pathways could be responsible. For example, TLR signaling and phagocytosis are hallmarks of macrophage-mediated innate immune responses to bacterial infections,21 and genes involved in Fc-dependent phagocytosis (eg, Lyn and Syk) have been found to be up-regulated by TLRs.22,23 Furthermore, both FcR- and TLR-mediated phagocytosis appear coupled—several TLR family members are known to localize to phagosomes where they can recognize molecules specific to pathogens and mediate inflammatory signaling.24,25 Perhaps LPS and autoantibody presented by the platelets to the THP-1 cells use shared components that synergistically increase signaling events and maximally stimulate macrophage phagocytosis.

In summary, LPS together with IgG bound to platelets significantly enhances Fc-mediated platelet phagocytosis by mononuclear phagocytes. These results suggest that infectious agents in combination with antiplatelet antibodies could affect platelet destruction in vivo, which may be at least one explanation of why thrombocytopenia worsens in some patients with AITP during infections and, alternatively, resolves in other patients with AITP who are treated with bacterial eradication therapy.

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Authorship

Contribution: J.W.S. designed research and wrote the first draft; R.A. designed and performed research, analyzed data, and corrected the draft; M.K. and E.R.S. performed research and analyzed data; and J.F. designed research, contributed human samples, analyzed data, and corrected the draft.

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