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. Opsonization 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)

Introduction

Autoimmune thrombocytopenic purpura (AITP) is a bleeding disorder caused by autoantibodies that opsonize platelets and enhance their destruction by FcR-mediated phagocytosis within the spleen.[1,2] Although both acute and chronic forms can be distinguished, acute AITP primarily affects children and often occurs after a viral or bacterial infection.[1,2] Furthermore, viral-specific antibodies with cross-reactivity against platelets have been identified in children with acute AITP[3,4] and in patients with HIV-related AITP,[4,5] suggesting infections may play a role in AITP pathogenesis. On the other hand, in some patients with chronic AITP, infections are associated with an exacerbation of thrombocytopenia, and this has also been demonstrated in a mouse model.[6] Alternatively, eradication of the Gram-negative bacterium *Helicobacter pylori* in patients with AITP increases platelet counts, although this has not been observed in some studies.[7-10] It is possible that, in susceptible individuals, infectious agents in the presence of antiplatelet antibodies affect platelet-monocyte interactions and alter platelet destruction.

Pathogens are first encountered by Toll-like receptors (TLRs) on professional phagocytes.[11,12] TLRs are germ line–encoded proteins that bind a variety of infectious molecular structures and are critical for stimulating innate immune mechanisms.[11,12] TLRs are also expressed on many cell types including platelets,[13-17] and platelet TLR4 expression at least appears responsible for mediating lipopolysaccharide (LPS)–induced thrombocytopenia in vivo.[15,16] To determine how bacterial agents modulate platelet destruction, we used flow cytometry to investigate whether LPS affects opsonized-platelet phagocytosis by mononuclear cells. The results demonstrate that LPS can synergize with IgG autoantibodies and significantly enhance platelet phagocytosis, suggesting at least one mechanism of how bacterial products may enhance platelet destruction in vivo.

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 described[18]; 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

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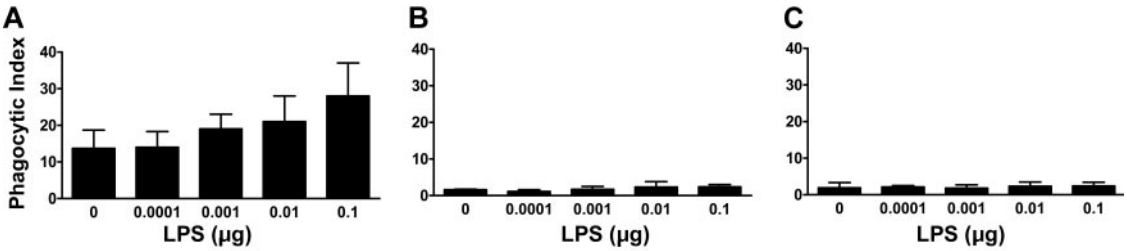

Figure 1. Effect of the indicated additions of LPS on the phagocytosis of (A) W6/32-opsonized, (B) nonopsonized, and (C) W6/32 F(ab′)$_2$ fragment-bound CM-G–labeled human platelets by THP-1 cells. The data are presented as the mean phagocytic index (± SD) from 10 (A-B) and 5 (C) independent experiments and were calculated by the following formula: median channel FL1 fluorescence at 37°C/median channel FL1 fluorescence at 4°C. Statistical significance in panel A by a 1-way ANOVA was $P < .001$.

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 1×10^7 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 5×10^6 THP-1 cells with 250×10^6 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 200*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 FACSort 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 0°C.

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

Compared with nonopsonized platelets, when W6/32-opsonized platelets were incubated with THP-1 cells, a significant ($P < .001$) increase in intracellular platelet fluorescence occurred (0 value from Figure 1A compared with 0 value from Figure 1B). In contrast, LPS-bound but nonopsonized platelets did not increase THP-1 intracellular platelet fluorescence (Figure 1B). If the LPS-bound platelets were, however, opsonized with W6/32, a significant ($P < .001$) and synergistic enhancement of opsonized-platelet phagocytosis occurred (Figure 1A). The synergistic LPS/antibody enhancement of phagocytosis was Fc specific because THP-1 phagocytosis was abrogated when the platelets were bound with W6/32 F(ab′)$_2$ fragments (Figure 1C). This suggests that LPS bound to platelets in the presence of antiplatelet antibody significantly increases platelet phagocytosis by human mononuclear cells. The binding of bacterial products such as LPS to platelets has been demonstrated to mediate several physiological processes including platelet aggregation and platelet-monocyte interactions and may also play a role in atherogenesis.[19,20] To our knowledge, however, this is the first demonstration of a synergistic effect between platelet-bound LPS and antiplatelet antibodies in enhancing platelet phagocytosis.

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

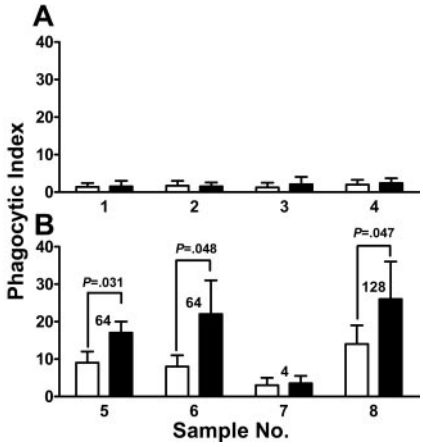

Figure 2. THP-1 phagocytosis of CM-G–labeled platelets. The platelets were incubated with either (A) autoantibody-negative sera samples 1 to 4 or (B) autoantibody-positive sera samples 5 to 8 and either no LPS (□) or 0.1 μg LPS (■) and incubated with THP-1 cells. The data are presented as the mean phagocytic index (± SD) from 4 independent experiments and were calculated as in Figure 1. The numbers on the x-axes refer to the human serum sample number, and IgG antiplatelet autoantibody titers in panel B are shown above the bars for each sera sample. Statistical significance (*P* values) by a paired *t* test is shown.

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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