Exacerbation of autoantibody-mediated thrombocytopenic purpura by infection with mouse viruses

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Antigenic mimicry has been proposed as a major mechanism by which viruses could trigger the development of immune thrombocytopenic purpura (ITP). However, because antigenic mimicry implies epitope similarities between viral and self antigens, it is difficult to understand how widely different viruses can be involved by this sole mechanism in the pathogenesis of ITP. Here, we report that in mice treated with antiplatelet antibodies at a dose insufficient to induce clinical disease by themselves, infection with lactate dehydrogenase-elevating virus (LDV) was followed by severe thrombocytopenia and by the appearance of petechiae similar to those observed in patients with ITP. A similar exacerbation of antiplatelet-mediated thrombocytopenia was induced by mouse hepatitis virus. This enhancement of antiplatelet antibody pathogenicity by LDV was not observed with F(ab′)2 fragments, suggesting that phagocytosis was involved in platelet destruction. Treatment of mice with clodronate-containing liposomes and with total immunoglobulin G (IgG) indicated that platelets were cleared by macrophages. The increase of thrombocytopenia triggered by LDV after administration of antiplatelet antibodies was largely suppressed in animals deficient for γ-interferon receptor. Together, these results suggest that viruses may exacerbate autoantibody-mediated ITP by activating macrophages through γ-interferon production, a mechanism that may account for the pathogenic similarities of multiple infectious agents. (Blood. 2004; 104:2102-2106) © 2004 by The American Society of Hematology

Introduction

Acute immune thrombocytopenic purpura (ITP) is a common disease of suspected autoimmune origin that often develops in the course of a viral infection, especially in children. In affected patients, platelets opsonized with autoantibodies are destroyed through phagocytosis by cells of the reticuloendothelial system, leading to clinical symptoms such as petechiae or, in more severe cases, bleedings. Widely different viruses, including Epstein-Barr virus, influenza, varicella zoster virus, cytomegalovirus, mumps, parvovirus, and rubella virus have been suspected as potential triggers of the disease. Molecular mimicry between epitopes shared by infectious agents and platelets, followed by spreading of the autoimmune response to new determinants, has been proposed as an explanation for the etiologic role of viruses in ITP. Interestingly, proinflammatory cytokines, such as γ-interferon (IFN-γ) have been found at higher levels in patients with ITP when compared with healthy individuals. Although the role of viruses in the pathogenesis of ITP is not disputable, it is, however, unclear how viruses with very divergent antigenic determinants can induce a similar autoimmune response focused to platelet epitopes through molecular mimicry.

So far, very few animal models have been developed that allow for an experimental approach of ITP. Mechanisms of platelet destruction may be analyzed in mice by using polyclonal antiplatelet antibodies at a dose insufficient to induce clinical disease by themselves, infection with lactate dehydrogenase-elevating virus (LDV) was followed by severe thrombocytopenia and by the appearance of petechiae similar to those observed in patients with ITP. A similar exacerbation of antiplatelet-mediated thrombocytopenia was induced by mouse hepatitis virus. This enhancement of antiplatelet antibody pathogenicity by LDV was not observed with F(ab′)2 fragments, suggesting that phagocytosis was involved in platelet destruction. Treatment of mice with clodronate-containing liposomes and with total immunoglobulin G (IgG) indicated that platelets were cleared by macrophages. The increase of thrombocytopenia triggered by LDV after administration of antiplatelet antibodies was largely suppressed in animals deficient for γ-interferon receptor. Together, these results suggest that viruses may exacerbate autoantibody-mediated ITP by activating macrophages through γ-interferon production, a mechanism that may account for the pathogenic similarities of multiple infectious agents. (Blood. 2004; 104:2102-2106) © 2004 by The American Society of Hematology

Materials and methods

Animals

Specific pathogen-free CBA/Ht, BALB/c nu/nu female and 129/Sv female mice were bred at the Ludwig Institute for Cancer Research by G. Warner and used when 8 to 12 weeks old. IFN-γ receptor–deficient mice (G129), received courtesy of F. Brombacher (Max Planck Institute for Immunobiology, Freiburg, Germany), were reared in the same manner as the 129/Sv rats or in mice. (NZW × BXSB)F1 mice spontaneously produce antiplatelet autoantibodies, leading to thrombocytopenia in the context of a complex autoimmune syndrome dissimilar to organ-specific ITP. Production of antiplatelet autoantibodies through molecular mimicry may be studied by immunizing mice with rat platelets, but the resulting thrombocytopenia is at best moderate and very transient in normal mice. Here, we report on the effect of infection with the common mouse viruses lactate dehydrogenase-elevating virus (LDV) and mouse hepatitis virus (MHV), on platelet counts in mice with preexisting antiplatelet autoantibodies. Infection triggers a clinical disease similar to childhood acute ITP through mechanisms that do not rely on autoantibody production by molecular mimicry but rather on activation of phagocytic cells after secretion of IFN-γ.
mice, from which they were initially derived by S. Huang and M. Aguet. Some mice were purchased from B&K Universal (Hull, United Kingdom). Spleens were surgically removed after anesthesia with ketamine hydrochloride (Imalgene 500; 1.25 mg/mouse; Merial Toulouse, France) and medetomidine hydrochloride (Domitor; 25 µg/mouse; Orion Pharma, Espoo, Finland), followed by atipamezole hydrochloride (Antisedan; 125 µg/mouse; Orion Pharma).

**Virus**

Mice were infected by intraperitoneal injection of approximately 2 × 10^3 50% infectious doses (ID_{50}) of LDV (Riley strain; ATCC, Rockville, MD) in 200 µL saline or of approximately 1 × 10^3 50% tissue culture infectious doses (TCID_{50}) of MHV-A59 grown in NCTC 1469 cells.

**Platelet counts**

Blood was collected from the retroorbital plexus of ether-anesthetized mice with the use of Unopette pipettes and kits (Unopette microllection system; Becton Dickinson, Franklin Lakes, NJ). Platelets were counted by microscopy with an improved Neubauer hemacytometer (Marienfeld, Germany).

**Antibodies**

Polyclonal antiplatelet antibodies were obtained after subcutaneous immunizations of a rabbit every 2 weeks with approximately 10^8 BALB/C mouse platelets, once with complete Freund adjuvant, then with incomplete Freund adjuvant. Immunoglobulin G (IgG) was isolated by treatment with acridine lactate (Rivanol; Chinosol, Seelze, Germany) and precipitation with half-saturated ammonium sulfate. F(ab')_2 fragments were prepared by pepsic digestion, followed by purification by chromatography on Ultrogel AcA 4–4 and precipitation with ammonium sulfate. Rat anti–mouse CD41 (integrin α_{IIb}β_{3} chain) monoclonal antibody was obtained from BD PharMingen (San Diego, CA). F96A9K1 is a monoclonal antiplatelet IgM autoantibody and N1 a monoclonal antiplatelet IgG2a autoantibody derived from (NZW × BXSB)F1 mice. Control monoclonal antibodies were rat IgG1 (LO-DNP1 from LO/IMEX, Université catholique de Louvain, Brussels, Belgium), mouse IgG2a (A6202F4), and mouse IgM (M444) of irrelevant specificity. Human IgG was Gamagard (Baxter, Lessons, Belgium). In vivo administration of antibodies was by the intraperitoneal route.

**Liposome preparation**

Clodronate-containing liposomes were prepared following the method described by Van Rooijen. Briefly, 86 mg phosphatidylcholine (Sigma, Bornem, Belgium) at 100 mg/mL in chloroform was added to 8 mg cholesterol (Sigma) in 10 mL chloroform. After evaporation, the lipidic film was dispersed by rotation for 10 to 15 minutes in 10 mL phosphate-buffered saline (PBS) alone or containing 2.5 g clodronate (dichloromethylene-bisphosphonate; kindly given by Boehringer Mannheim GmbH, now Roche Diagnostics GmbH, Germany). Liposomes were sonicated for 3 minutes at 50 W, then washed with PBS, and resuspended in 4 mL PBS. Mice were treated by intravenous injection with 200 µL liposomes.

**Results**

**Exacerbation of antibody-mediated thrombocytopenia by viral infection**

To determine the effect of a viral infection on experimental ITP, we administered LDV and rabbit anti–mouse platelet polyclonal antibodies to normal mice. As shown in Figure 1A, when administered alone, both the virus and the antibody, at the selected dose, induced a moderate thrombocytopenia in CBA/Ht animals, whereas in mice with other genetic backgrounds, such as BALB/C, there were no consequences (not shown). In contrast, a sharp drop in platelet counts was observed in mice that received both the virus and the antiplatelet antibody (P = .0286 for mice treated with antiplatelet antibody and LDV versus antibody or virus alone). A similar exacerbation of antiplatelet antibody–mediated thrombocytopenia was observed in mice infected with MHV 1 day before antiplatelet antibody administration (P = .0079 for mice treated with antiplatelet antibody and MHV versus antibody or virus alone; Figure 1B). Analysis of this effect of viral infection was performed after LDV inoculation in all subsequent experiments. LDV infection was also found to enhance antiplatelet antibody–mediated thrombocytopenia in BALB/c nu/nu mice (P = .0286 for mice treated with antiplatelet antibody and LDV versus antibody or virus alone; Figure 2B). This effect of the virus led to a clinical picture reminiscent of purpura developing in patients (Figure 2A). Numerous petechiae could indeed be detected on mice that had received both the virus and the antiplatelet antibody but not in animals that were only infected or that were treated with the antibody alone.

LDV similarly worsened the thrombocytopenia induced by a rat monoclonal antibody recognizing mouse CD41 (platelet glycoprotein IIb; BD PharMingen, San Diego, CA) and by an IgG2a (N1) and an IgM (F96A9K1) monoclonal autoantibodies derived from (NZW × BXSB)F1 mice that spontaneously develop autoantibody-mediated thrombocytopenia (as shown for typical experiments in Figure 3A). Moreover, kinetics experiments indicated that the effect of LDV was transient (Figure 3B). A sharp enhancement of autoantibody-mediated thrombocytopenia was indeed observed in mice that had been infected the same day, and 1 or 2 days before antiplatelet antibody inoculation (P = .0079), but not in animals that were infected for 4 days or more. Infection 1 or 2 days after antiplatelet antibody administration also resulted in an increase of thrombocytopenia (data not shown).
A and B represent independent experiments. Mined which part of the immunoglobulin was required in this phenomenon. Administration of F(ab')2 fragments of antiplatelet polyclonal antibody was not followed by the development of disease, even in infected animals, in contrast to intact immunoglobulin (significant difference between infected mice that received antiplatelet IgG or F(ab')2: \( P = 0.0286 \); Figure 4). This suggests that phagocytosis is involved in LDV-enhanced thrombocytopenia. This hypothesis was tested by administration of clodronate-containing liposomes to LDV-infected mice, a treatment known to suppress macrophages with phagocytic activity10 and, therefore, to cure experimental autoantibody-mediated blood autoimmune diseases that are mediated by phagocytosis.6,11,12 Administration of these clodronate-containing liposomes resulted in a complete prevention of the increased pathogenicity of both polyclonal and monoclonal antiplatelet antibodies observed in LDV-infected mice (significant difference for all antibodies between mice treated with PBS- and clodronate-containing liposomes: \( P = 0.0286 \); Figure 5A). The hypothesis that LDV enhances thrombocytopenia through Fc receptor–mediated phagocytosis was further supported by administration of total human IgG to mice. This treatment has previously been shown to inhibit in vivo Fc receptor–mediated phagocytosis of autoantibody-coated red blood cells and to subsequently decrease the resulting hemolytic anemia.13 Similarly, LDV-enhanced antibody-mediated thrombocytopenia was largely suppressed by administration of total human IgG (significant difference between infected mice that received antiplatelet antibody and treated with total human IgG, or untreated: \( P = 0.0286 \); Figure 5B). However, the major site of this increased phagocytosis of antibody-coated platelets was not the spleen, because administration of antiplatelet antibodies to LDV-infected splenectomized mice and mock-operated mice induced a thrombocytopenia that was not quite significantly different (\( P = 0.0571 \); Figure 6).

Because IFN-\( \gamma \) is a potent activator of macrophage function, a possible role for this cytokine in LDV-induced enhancement of antibody-mediated thrombocytopenia was investigated by infecting mice deficient for the receptor of this cytokine (G129). Although the thrombocytopenia induced by the virus alone was not significantly different in these animals and in their normal counterparts, the aggravating effect of LDV on the pathogenicity of antiplatelet antibodies was largely decreased in mice unable to respond to IFN-\( \gamma \). This suppression of LDV-enhanced thrombocytopenia in mice deficient for IFN-\( \gamma \) receptor was observed after administration of both polyclonal anti–mouse platelet rabbit IgG (Figure 7A) and monoclonal rat anti–mouse CD41 antibody (Figure 7B).

Mechanisms involved in LDV-enhanced antibody-mediated thrombocytopenia

To further analyze the mechanisms by which a viral infection may exacerbate antibody-mediated thrombocytopenia, we first determined which part of the immunoglobulin was required in this process. It was previously shown that the \( \alpha \) chain of the platelet Fc receptor, the FcR \( \alpha \) (CD63), is a high-affinity receptor for anti-CD41 antibodies and that this receptor is expressed on bone marrow-derived hematopoietic cells.14 Although the thrombocytopenia induced by the virus alone was not significantly different from normal and IFN-\( \gamma \)-deficient mice, the exacerbation of LDV-enhanced thrombocytopenia was significantly increased in mice expressing the FcR \( \alpha \) chain (G129) and this increase was not observed in normal and IFN-\( \gamma \)-deficient mice (\( P = 0.0286 \); Figure 6).

Because IFN-\( \gamma \) is a potent activator of macrophage function, a possible role for this cytokine in LDV-induced enhancement of antibody-mediated thrombocytopenia was investigated by infecting mice deficient for the receptor of this cytokine (G129). Although the thrombocytopenia induced by the virus alone was not significantly different in these animals and in their normal counterparts, the aggravating effect of LDV on the pathogenicity of antiplatelet antibodies was largely decreased in mice unable to respond to IFN-\( \gamma \). This suppression of LDV-enhanced thrombocytopenia in mice deficient for IFN-\( \gamma \) receptor was observed after administration of both polyclonal anti–mouse platelet rabbit IgG (Figure 7A) and monoclonal rat anti–mouse CD41 antibody (Figure 7B).

**Figure 2.** Thrombocytopenic purpura induced by LDV infection in nude mice. (A) Global clinical picture and skin detail of BALB/c nu/nu mice 1 day after administration of IgG from polyclonal anti–mouse platelet rabbit serum (anti-plt Ab) (3 \( \mu \)g; iii-iv) or control rabbit IgG (i-ii) and of LDV (ii, iv) or saline (i, iii). Photographs were taken with a Nikon Coolpix 4500 (Nikon, Tokyo, Japan). (B) IgG (3 \( \mu \)g) from polyclonal anti–mouse platelet rabbit serum or control rabbit IgG was injected into groups of 4 BALB/c nu/nu mice with LDV infection or administration of saline. Platelets were counted 2 days later (\( \bullet \) represents each individual datum; \( \circ \), means \( \pm \) SEM). Panels A and B represent independent experiments.
Discussion

Our results indicate that LDV, in addition to inducing a transient thrombocytopenia by itself, can dramatically enhance thrombocytopenia that is triggered concomitantly to the infection by antiplatelet antibodies. This effect of the virus on antiplatelet antibody–induced thrombocytopenia does not require T lymphocytes, because it is observed in nude mice. Therefore, it is not caused by new antibody production in response to infection, because such antibody responses are T-cell dependent and do not start before 4 days after infection, and antigenic mimicry cannot explain this virally induced platelet drop. Because LDV may enhance phagocytosis, it could be postulated that the virus induces thrombocytopenia by this mechanism. Indeed, treatment with clodronate-containing liposomes indicated that the enhancing effect of LDV on autoantiplatelet antibody-dependent thrombocytopenia involves phagocytosis exclusively. Moreover, inhibition of this thrombocytopenia with total IgG indicated that LDV-enhanced phagocytosis of opsonized platelets was at least partly mediated by Fc receptors expressed on activated macrophages. Which type of receptors is involved and whether infection leads to an increase of the expression of these receptors remains to be determined. Interestingly, the moderate and transient thrombocytopenia induced by LDV infection in the absence of antiplatelet antibody administration was only partially prevented by treatment with clodronate-containing liposomes (data not shown). This suggests that different mechanisms may be involved in the thrombocytopenia induced by the virus alone or in the presence of antiplatelet antibodies.

Because macrophage functions, and especially phagocytosis, are enhanced by IFN-γ, a cytokine produced in the course of LDV infection, this cytokine was suspected as a mediator of LDV-induced enhancement of antiplatelet antibody pathogenicity. The causative role of IFN-γ was shown in mice genetically unable to respond to this cytokine. The transient effect of LDV on antiplatelet antibody pathogenicity may thus be explained by the peculiar kinetics of immune modulation observed after infection with this virus. Indeed, IFN-γ production after LDV infection reaches a peak 18 hours after virus inoculation, and then rapidly decreases. In addition to IFN-γ, we cannot exclude that other cytokines, including interleukins 2 and 12 and macrophage colony-stimulating factor (M-CSF) may also be involved in this virally induced activation of macrophage functions.

Figure 4. Requirement of immunoglobulin Fc portion in LDV-enhanced antibody-mediated thrombocytopenia. Groups of 4 BALB/c mice received either 3 μg control or anti–mouse platelet (anti-plt) rabbit polyclonal IgG or 6 μg anti–mouse platelet rabbit polyclonal F(ab′)2 1 day after LDV infection or administration of saline. Platelets were counted 2 days after antibody administration. ○ represents each individual datum; •, means ± SEM.

Figure 5. Role of phagocytosis in LDV-mediated thrombocytopenia. (A) Antiplatelet antibodies (polyclonal rabbit IgG, 1 μg/mouse; monoclonal N1 IgG2a and F96A9K1, 100 μg/mouse) were administered 1 day after LDV infection in groups of 4 CBA/Ht mice. Animals were treated with PBS-containing or clodronate-containing liposomes (200 μL) as indicated 36 hours before antibody administration. Control mice were infected but received saline instead of antiplatelet antibody. Platelets were counted 2 days after antibody administration (results shown as means ± SEM). Experiment with polyclonal antibody was independent from experiment with monoclonal antibodies. (B) IgG (3 μg) purified from polyclonal anti–mouse platelet rabbit serum (anti-plt Ab) or control rabbit IgG was administered 1 day after LDV infection in groups of 4 CBA/Ht mice. Animals were treated with 5 daily doses of 4 mg total human IgG, where indicated, starting 3 days before antibody administration. Platelets were counted 1 day after antibody administration. ○ represents each individual datum; •, means ± SEM.
It is not easy to understand how the antigenically different pathogens that have been suspected to trigger childhood acute ITP may lead to autoantibody-mediated thrombocytopenia through antigenic mimicry. However, because macrophage activation and IFN-γ production may be induced by distinct viruses, with diverse kinetics, exacerbation of antibody-mediated thrombocytopenia through enhanced phagocytosis may explain the similar consequences on platelet counts observed after infection of patients with widely different pathogens. This hypothesis is further supported by our observation that MHV, which also triggers IFN-γ production, has an enhancing effect on antiplatelet pathogenicity similar to that found after LDV infection. We can thus hypothesize that the development of childhood acute ITP requires 2 successive events: first, a modification of the potential recognition of platelets by phagocytic cells that might be due to autoantibody production, and that remains clinically silent, and then a triggering of thrombocytopenia as a consequence of IFN-γ-enhanced phagocytosis by macrophages in the course of infection with one of many different viruses. Therefore, targeting this cytokine-induced macrophage activation might be another alternative for new treatments of autoimmune blood diseases that follow viral infections.

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References

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