Original Research

Immunoglobulin M antiplatelet autoantibodies from mice immunized with rat platelets induce thrombocytopenia and platelet function impairment

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Abstract

Antiplatelet monoclonal autoantibodies (mAbs) were derived from CBA mice immunized with rat platelets. Two IgM antiplatelet mAbs were further analyzed. L1C43 mAb bound a 150–160 kDa antigen, recognized activated platelets better than resting ones and impaired platelet adhesion, but not clot retraction. L1H31 mAb recognized a ±95 kDa molecule, bound similarly activated and resting platelets and did not modify platelet adhesion, but inhibited clot retraction. Both mAbs induced *in vivo* thrombocytopenia most likely through phagocytosis of opsonized platelets. These autoreactive antibodies can thus induce both platelet destruction and impairment of their function. They are reflective of autoantibodies found in human patients and may serve for further analysis of antiplatelet autoantibody pathogenicity and mechanisms of autoimmune disease.

Keywords: platelets, monoclonal autoantibodies, thrombocytopenia, phagocytosis, platelet function, immune thrombocytopenic purpura

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Introduction

Immunization of normal CBA mice with rat platelets results in the development of an autoantibody response leading to moderate and transient thrombocytopenia.[1] Analysis of polyclonal autoantibodies eluted from the platelets of these immunized mice indicated that they recognize epitopes shared by mouse and rat platelets contained at least in a ±150 kDa protein that co-migrates with platelet glycoprotein Ib (GPIb, CD42b). In addition, a ±95 kDa protein with a migrating pattern similar to GPIIIa (CD61) was also recognized. These polyclonal autoantibodies can induce thrombocytopenia when injected into naive animals.[1] This model is arguably the closest model in the mouse for human immune thrombopenic purpura and post-transfusion purpura.

Although antiplatelet monoclonal antibodies (mAbs) that are of great interest for analyzing platelet surface molecule functions have already been reported,[2,3] these mAbs were derived after xenogenic immunization and are therefore not fully reflective of antiplatelet autoantibodies. Mouse monoclonal antiplatelet autoantibodies have also been derived from (NZBxBXSB)F1 animals.[4] These mAbs have been proven very useful to analyze the mechanisms of opsonized platelet destruction.[5,6] However, (NZBxBXSB)F1 mice develop a complex systemic autoimmune syndrome that probably involves mechanisms different from those responsible for the autoantibody response elicited by CBA mouse immunization with rat platelets. To gain further insight into the characteristics of the latter autoimmune response, mAbs recognizing mouse platelets were derived from immunized CBA animals. Two IgM mAbs were especially analyzed and their *in vitro* and *in vivo* pathogenicity was determined.

Materials and methods

Animals

Specific pathogen-free CBA/Ht female mice were bred at the Ludwig Institute for Cancer Research by G Warnier and CBA/Ca mice were purchased from Harlan. They were used when 8–12 weeks old. NMRI mice and Wistar rats were bred in our local facility by J-P Dehoux. The project was approved by the local commission for animal care.

Immunization

Rat platelets were prepared by successive centrifugations as described previously.[1] Mice were immunized first by intraperitoneal injection of 10^8 rat platelets in 0.5 mL saline, followed by weekly injections of 0.5×10^8 platelets.

Antibodies

Fluoresceinated rat anti-mouse CD41 (integrin α_{IIb} chain) mAb and fluoresceinated rat anti-mouse CD62P (P-selectin) mAb were obtained from BD PharMingen (San Diego, CA, USA). Fluoresceinated rat anti-mouse kappa chain mAb (LO-MK1) was from LO/IMEX (Université catholique de Louvain, Brussels, Belgium). Control mAb was a mouse IgM (M444) of irrelevant specificity. Rat anti-mouse CD41 (integrin α_{IIb} chain) mAb, mouse anti-human CD42b (platelet glycoprotein Ib) and hamster anti-mouse CD61 (platelet glycoprotein IIIa) were obtained from BD PharMingen.

Enzyme-linked immunosorbent assay

Reactivity of antibodies with rat, mouse or human platelets, and with bovine serum albumin (BSA) was analyzed by enzyme-linked immunosorbent assay (ELISA) as described in reference.[1] Briefly, plates coated with rat or mouse platelets, or with BSA, were incubated with samples to be tested followed by rat IgG2a anti-mouse k-chain mAb conjugated to horseradish peroxidase (LO-MK1) and by 3,3′,5,5′-tetramethylbenzidine (Thermo Fisher Scientific, Rockford, IL, USA).

Flow cytometry (fluorescence-activated cell sorting)

Mouse mAb binding to platelets was analyzed by flow cytometry after detection with fluoresceinated rat IgG2a anti-mouse k-chain mAb (LO-MK1) as described.[1] Platelet activation was achieved by a five-minute incubation with 8 U/mL thrombin (Sigma-Aldrich, St Louis, MO, USA).

Western blots

Western blots were performed as described previously[1] with lysed platelet proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 8% or 10% gels under non-reducing conditions, transferred onto nitrocellulose membranes and incubated with antiplatelet mAbs followed by rat anti-mouse kappa chain mAb conjugated to horse-radish peroxidase then by ECL™ Western blotting detection reagents (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK).

Platelet counts

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

Liposomes

Clodronate-containing liposomes were prepared following the method described previously.[7]

In vitro phagocytosis assay

NMRI platelets (3×10^8/mL in buffered saline glucose citrate [BSGC buffer][1]) were incubated for one hour at room temperature with 3 μmol/L 5-chloromethylfluorescein diacetate (CellTracker Green™ CMFDA; Molecular Probes, Eugene, OR, USA), washed twice with BSGC, incubated for one hour at room temperature with either polyclonal rabbit anti-mouse platelet antibody or control and anti-platelet IgM Abs (0.4 μg/10^6 platelets), and washed again. Peritoneal cells (3×10^6/mL) were incubated for three hours at 37°C in Iscove's Modified Dulbecco's medium supplemented with 10% heat-inactivated fetal calf serum. After washing to remove non-adherent cells, 2×10^7 opsonized CMFDA (5-chloromethylfluorescein diacetate)-labeled platelets/mL were added for two hours 30 minutes at 37°C. Cells were then washed twice in phosphate-buffered saline (PBS), harvested with Trypsin-EDTA (Lonza, Verviers, Belgium), fixed with 1.25% paraformaldehyde and analyzed by flow cytometry.

Clot retraction assay

One hundred microliter of platelet-rich plasma were added to 160 μL clotting buffer (5.3 mmol/L Na_2HPO_4, 12 mmol/L KH_2PO_4) followed sequentially by 5 μL pelleted erythrocytes, mAbs in 200 μL PBS and 50 μL thrombin (10.4 U/mL). Incubation was performed for approximately 30 min at 37°C in the presence of a glass rod.

Platelet adhesion and aggregation

Platelet adhesion and aggregation was measured with human platelets by Impact-R test (DiaMed, Cressier-sur-Morat, Switzerland),[8,9] following the manufacturer's instructions. Whole blood was anticoagulated for 30 min with sodium citrate (1 part 0.106 mol/L sodium citrate for 9 parts blood) in S-Monovette tubes (Sarstedt). mAb was added at 10 μg/mL for 30 min at room temperature. One hundred and thirty microliters of the sample was placed on a polystyrene plate with a standardized pipette and the bell housing containing the teflon cone was fitted on top. The program was immediately started, with a 15-s preincubation followed by the application of shear force (1800/s) for two minutes. Plates were washed and stained with May-Grünwald solution and the samples were analyzed with an inverted light microscope. Seven images were taken for each run and the average percentage of total area covered by platelets (SC %, measuring adhesion) and the average size of surface-bound objects (AS μm^2, measuring aggregation) were calculated from the three more representative images by the image analyzing system.

Statistical analysis

When appropriate, statistical analysis was performed using unpaired Student's *t*-test or non-parametric Mann-Whitney *U* test.

Results

Production of antiplatelet monoclonal antibodies

Hybridomas producing monoclonal antiplatelet antibodies were derived after fusions of 10^8 spleen cells from CBA/Ht or CBA/Ca mice immunized three times with rat platelets, with 2×10^7 SP120 or SP207 myeloma cells by conventional procedures. Three fusion experiments were performed. After the first fusion, 324 clones were first screened by ELISA on plates coated with mouse platelets and nine clones were derived for further analysis. One positive on 56 clones and four on 70 clones tested were obtained after the second and third fusions, respectively. The last of these fusions was performed after initial panning of spleen cells on plates coated with rat platelets. Of the 14 clones selected, nine were IgM, three were IgG2a and two were IgG2b.

After subcloning and purification twice with ammonium sulfate, antibodies secreted by these clones were tested again by ELISA on plates coated with mouse or rat platelets or with BSA (Figure 1). Eight of these mAbs were found to react stronger with platelets than with BSA. Their binding was similar with mouse (Figure 1a) and rat (Figure 1b) platelets. In addition, mAbs such as L1H31 and L1C43 bound human platelets as strongly as a mouse anti-human CD42b (Figure 1c). Interestingly, all these mAbs-recognizing platelets were of the IgM isotype. Little reaction of the other mAbs was observed in this assay, both with mouse and with rat platelets.

Binding of these mAbs to platelets was further investigated by fluorescence-activated cell sorting analysis using both resting and activated platelets. As positive controls, an anti-CD41 mAb recognized equally most resting and activated platelets, while an anti-CD62P mAb bound activated platelets only. Anti-CD42b and anti-CD61 commercial mAbs also strongly recognized platelets (geometric mean fluorescence after incubation with resting and activated platelets, respectively: control: 1.2 and 1.2; anti-CD42b: 44.5 and 13.9; anti-CD61: 51.2 and 180.2). As shown in Figure 2, all the mAbs that were positive by ELISA, with the exception of L5E54, reacted more with resting platelets than did a control IgM or the secondary rat antibody alone. This was also the case for L6B32 that was negative in ELISA. However, this binding to resting platelets of our IgM mAbs was much weaker than that of the control IgG anti-CD41 mAb. The binding of these IgM mAbs was strongly enhanced when activated platelets were used, with the exception of L1H31 and L6B32, which recognized similarly resting and activated platelets. For several mAbs, the fraction of activated platelets that were recognized was similar to that observed with an anti-CD62P mAb, whereas the fluorescence intensity was usually not as strong as with this control IgG mAb (not shown).

Antiplatelet mAb fine specificity was further analyzed in Western blots (Figure 3). For most mAbs, it was not possible to determine a clear recognition pattern (not shown). However, L1C43 mAb bound a $\pm$150–160 kDa band (Figure 3a). Anti-CD42b mAb recognized a band with a similar molecular weight. This specificity corresponded to the bulk of antibodies eluted from platelets of immunized

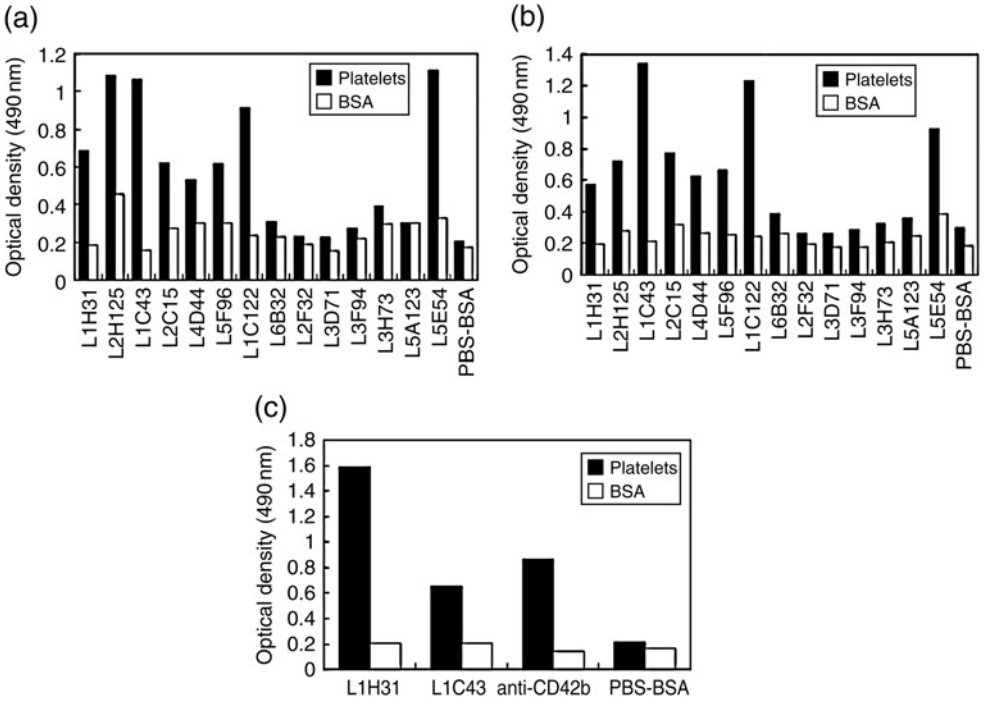

Figure 1 Detection of antiplatelet monoclonal autoantibodies (mAbs). Purified mAbs (10 μg/mL) were analyzed by enzyme-linked immunosorbent assay on plates coated with mouse (a), rat (b) or human (c) platelets (closed columns), or with bovine serum albumin (BSA) (open columns)

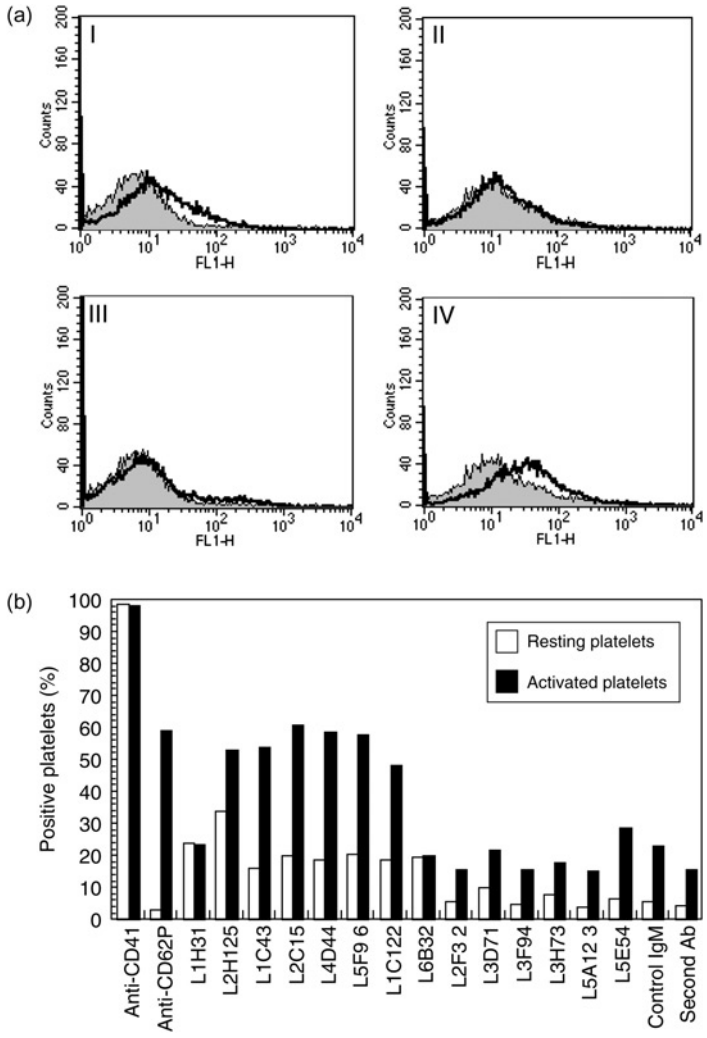

Figure 2 Binding of antiplatelet monoclonal autoantibodies (mAbs) on resting and activated platelets. Binding of mAbs was determined by fluorescence-activated cell sorting analysis on resting mouse platelets and on platelets activated by incubation with thrombin. (a) Binding of L1H31 (bold line, I and II) and of L1C43 mAbs (bold line, III and IV) on resting (I and III) or activated (II and IV) platelets. The gray area shows binding of a control IgM mAb. (b) Binding of antiplatelet mAbs on resting (open columns) and activated (closed columns) platelets, expressed as % of positive platelets with an arbitrary gate

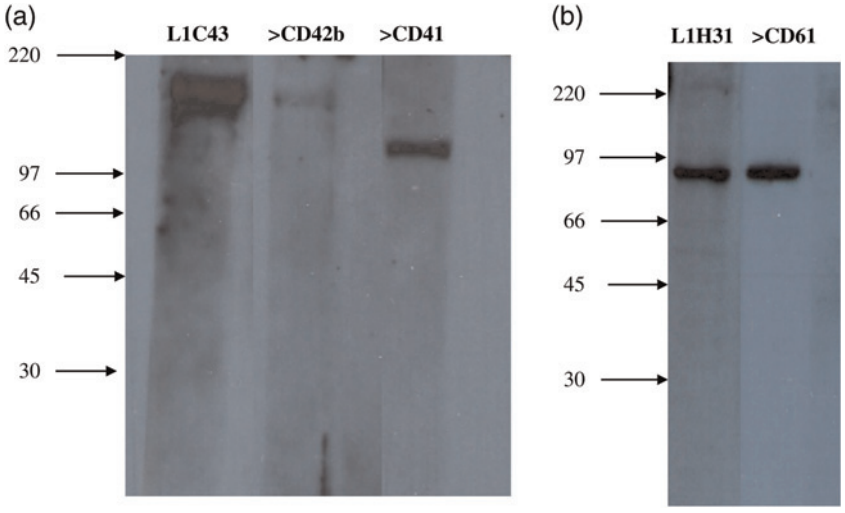

Figure 3 Western blot analysis of antiplatelet monoclonal autoantibodies (mAbs). Specificity of L1C43 (a) and L1H31 (b) mAbs was analyzed in Western blots loaded with mouse platelet lysates. Controls were provided by anti-CD42b, anti-CD41 and anti-CD61 mAbs (A color version of this figure is available in the online journal)

mice.[1] In contrast, L1H31 mAb recognized a ±95 kDa band (Figure 3b). A band with a similar migration pattern was bound by an anti-CD61 mAb. Antibodies with a similar specificity had also been observed in platelet eluates.[1] L1C43 and L1H31 mAbs were selected for further pathogenic analysis.

In vivo pathogenicity of antiplatelet mAbs

In vivo pathogenicity of L1C43 and L1H31 antiplatelet mAbs was determined by administration of 300 μg mAb per mouse. This treatment triggered a significant thrombocytopenia when compared with a control IgM, as soon as one day after mAb injection. Thrombocytopenia following L1C43 and L1H31 mAb treatment was similar (very significant statistical difference at one day after mAb administration: $P = 0.0013$ and 0.0087 for L1C43 and L1H31, respectively). It progressively resolved and platelet counts returned to normal levels four days after mAb administration (not shown). A similar result was found in three independent experiments.

Platelet phagocytosis by macrophages is a major mechanism leading to thrombocytopenia. To further determine whether this thrombocytopenia was induced by phagocytosis of platelets opsonized by the antiplatelet mAbs, mice were treated with clodronate-containing liposomes, a technique that has been widely used to deplete *in vivo* phagocytozing cells of the reticulo-endothelial system.[7,10–12] Therefore, such a treatment has been successfully used to determine the role of target cell trapping by macrophages in mouse models of diseases such as immune thrombocytopenia or anemia.[13–16] This treatment resulted in a prevention of thrombocytopenia induced by both mAbs (Figure 4; highly significant difference between PBS- and clodronate-containing liposomes: $P = 0.0017$ and 0.0018 for L1C43 and L1H31, respectively). A similar result was observed in two independent experiments.

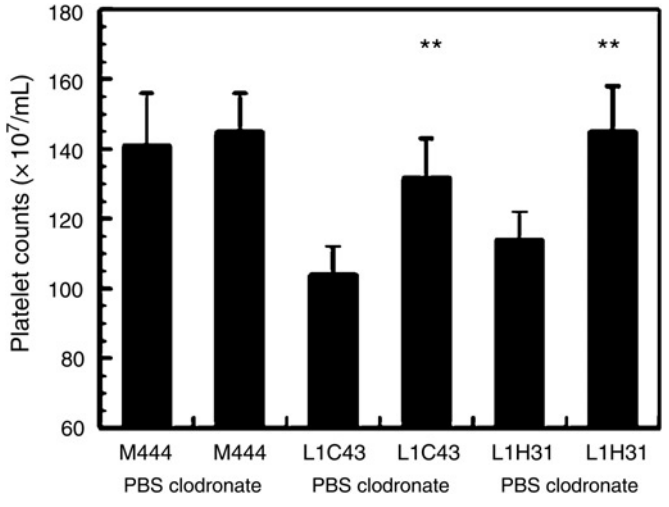

Figure 4 Role of phagocytosis in the pathogenicity of antiplatelet monoclonal autoantibodies (mAbs). Groups of five CBA mice were treated by intravenous injection with 200 μL either phosphate-buffered saline (PBS)- or clodronate-containing liposomes followed one day later by 300 μg mAb by intraperitoneal injection. Platelets counts were determined one day after mAb administration. Results are shown as means ± SD. **Very significant difference between PBS- and clodronate-containing liposomes

Phagocytosis of platelets incubated with antibodies was analyzed *in vitro*, by using CMFDA-labeled platelets. As shown in Figure 5, phagocytosis by macrophages was clearly enhanced when platelets were opsonized with polyclonal antibodies (very significant difference between mean geometric fluorescence intensity for platelets opsonized with polyclonal antibody versus platelets incubated with control IgM antibody, $P = 0.0043$). In contrast, although in some experiments, L1C43 and L1H31 IgM mAbs had a very slight enhancing effect on platelet phagocytosis when compared with a control IgM mAb, this effect was not reproducibly observed (Figure 5). Phagocytosis was not enhanced by the addition of complement (not shown).

In vitro impairment of platelet function by antiplatelet mAbs

In addition to the initiation of thrombocytopenia, antiplatelet mAbs could also impair some platelet functions. To analyze such an effect, the ability of L1C43 and L1H31 mAbs to inhibit clot retraction was measured (Figure 6).

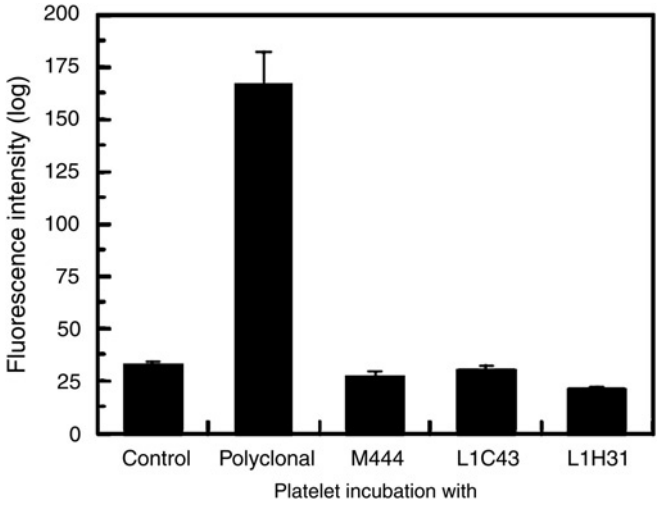

Figure 5 *In vitro* phagocytosis of opsonized platelets. Phagocytosis of CMFDA-labeled platelets by peritoneal macrophages was measured by flow cytometry after platelet incubation with control medium, control IgM (M444) or polyclonal L1C43 and L1H31 IgM antiplatelet antibodies. Results of 4–6 measurements are shown as means ± SD of geometric means of fluorescence intensity. CMFDA, 5-chloromethylfluorescein diacetate

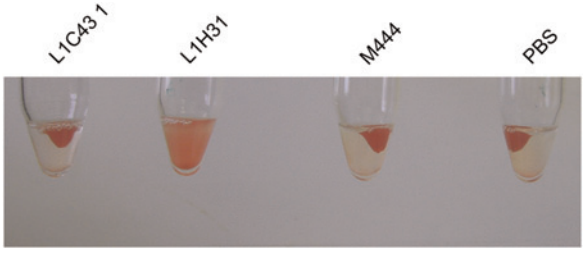

Figure 6 Inhibition of clot retraction by antiplatelet monoclonal autoantibodies (mAbs). Clot retraction was performed without antibody (phosphate-buffered saline) or in the presence of control IgM (M444) or L1C43 and L1H31 antiplatelet mAbs (200 μg/mL) (A color version of this figure is available in the online journal)

Clot retraction was completely inhibited by L1H31 mAb. In contrast, no inhibition was induced by L1C43 mAb or by a control IgM (M444). This result was obtained in three independent experiments.

The ability of L1C43 and L1H31 mAbs to impair platelet aggregation and adhesion was analyzed with Impact technology. As this technology has been developed for human platelets, these, rather than mouse platelets, were used for analysis. Indeed, in our hands, results obtained with mouse platelets with this method were not reproducible enough to be taken into account (data not shown). It was first determined by ELISA that both L1C43 and L1H31 mAbs effectively bind human platelets (Figure 1c). A compilation of results from four independent experiments performed with Impact (Table 1) indicated that platelet adhesion and aggregation were not inhibited by L1H31 mAb, when compared with PBS ($P = 0.3306$ and 0.4889 for SC and AS, respectively, for 250 μg/mL mAb) or with a similar dose (10 μg/mL) of control IgM ($P = 0.1880$ and 0.0926 for SC and AS, respectively). In contrast, pooled results from four independent experiments with L1C43 mAb showed a strong inhibition of platelet adhesion ($P = 0.0033$ and 0.0002 for 10 μg/mL mAb compared with PBS and control IgM, respectively), although aggregation was barely modified even with high-dose L1C43 mAb (250 μg/mL; $P = 0.4018$ and 0.0395 when compared with PBS and control IgM, respectively).

Discussion

Using a protocol of mouse immunization against rat platelets,[1] we have derived mAbs that recognized autoantigens expressed on mouse platelets and that should be representative of autoreactive antibodies secreted in this model of antiplatelet autoimmune disease. Although IgG2a and IgG2b antiplatelet mAbs were initially obtained, all those that were ultimately found to specifically bind platelet antigens were of the IgM isotype. It is not clear why antiplatelet IgG mAbs could not be derived, even after enrichment of spleen cells for B lymphocyte producing IgG2a Abs (not shown). Whereas it cannot be excluded that IgM-producing cells would better hybridize in our system, the difference with the isotypic distribution of Abs eluted from platelets might be due to a better affinity of IgGs that would then be over-represented in platelet-associated Igs, even if initially produced at much lower levels. All of our antiplatelet mAbs recognized rat as well as mouse platelet antigens. Those that were ultimately analyzed in more detail recognized human platelets as well.

Table 1 Inhibition of platelet adhesion and aggregation by mAbs

mAb	Dose (μg/mL)	*n**	SC (%)†	AS (μm²)‡
PBS	–	12	11.0 ± 3.4	45.8 ± 9.7
M444§	10	6	14.3 ± 2.7	70.7 ± 12.4
L1C43	10	4	4.7 ± 1.3	39.8 ± 9.4
	250	4	2.1 ± 1.0	51.0 ± 12.4
L1H31	10	2	11.0 ± 2.8	51.5 ± 7.8
	250	6	9.5 ± 2.1	42.5 ± 8.7

mAb, monoclonal autoantibody; PBS, phosphate-buffered saline; SC, surface coverage; AS, average size
*Number of measurements
†Surface coverage (adhesion), mean ± SD
‡Average size (aggregation), mean ± SD
§Control IgM

Although the specificity of these mAbs could not be definitely determined, probably due to their isotype, one of them (L1C43) recognized a ±150–160 kDa molecule, whereas the other (L1H31) bound a ±95 kDa antigen. Therefore, our two mAbs display a specificity similar to that of polyclonal autoantibodies eluted from platelets from CBA mice after rat platelet immunization that also bound antigens with these migrating patterns.[1] These molecular weights could correspond with platelet GPIb and GPIIIa, which were recognized by anti-CD42b and anti-CD61 commercial mAbs, respectively. However, it was not possible to confirm their specificity by immunoprecipitation experiments, possibly due to a weak affinity of our mAbs.

L1C43 mAb was able to inhibit *in vitro* platelet adhesion, whereas L1H31 mAb could suppress clot retraction. These results indicate that our antiplatelet autoantibodies can impair platelet functions, which may have important consequences for their pathogenicity. Moreover, because the GPIIb/IIIa complex interacts with fibrinogen and is involved in clot retraction,[17] this might further suggest that L1H31 mAb could recognize GPIIIa. On the other hand, blocking of von Willebrand factor binding to platelet glycoprotein Ib results in decreased adhesion,[8] which is consistent with the effect of L1C43 mAb. Thus, GPIb might be the target of L1C43 mAb.

However, some discrepancies remain. Most of our antiplatelet mAbs were found by flow cytometry analysis to recognize activated platelets more efficiently than resting ones. Platelet activation is usually associated with an enhanced expression of GPIIb/IIIa complexes on platelet surface and with a decrease in the expression of the GPIb/IX complex,[18] a result that we could confirm using commercial mAb. In contrast, we found an increased binding to activated platelets of the L1C43 mAb that bound a platelet antigen with similar molecular weight than GPIb. Although the reason of this discrepancy remains undetermined, it might be due to conformational changes in GPIb that would result in enhanced exposure of the epitope recognized by L1C43 mAb. However, we cannot exclude that antigens bound by our mAbs are distinct from GPIb and GPIIIa. Nevertheless, this observation indicated that some autoantibodies may better bind to activated than to resting platelets, which could result in an increased pathogenicity, since they may lead to a more efficient destruction of these activated platelets.

Administration of the two mAbs triggered a similar thrombocytopenia, independently from their specificity. Interestingly, thrombocytopenia has already been reported after administration of both anti-GPIIb/IIIa and anti-GPIb/IX/V mAbs.[19] However, these rat mAbs were of the IgG isotype and induced thrombocytopenia through distinct mechanisms. In both cases, platelet destruction after treatment with our mAbs was reduced after treatment with clodronate-containing liposomes that suppress phagocytozing macrophages.[10–16] Although it was not possible to

demonstrate *in vitro* phagocytosis of IgM-opsonized platelets, this observation points toward phagocytosis as a major mechanism involved in thrombocytopenia triggered by L1C43 and L1H31 antiplatelet mAbs. However, since both mAbs were of the IgM isotype, this phagocytosis could not be mediated by Fcγ receptors. In addition, complement binding did not likely play a role, since neither addition of complement in the *in vitro* experiment, nor *in vivo* complement depletion with cobra venom factor, modulated platelet destruction (not shown). It may therefore be postulated that other *in vivo* phenomena, such as formation of large aggregates of platelets and autoantibodies that would be easily trapped by macrophages without involvement of Fc or complement receptors, may be responsible for the thrombocytopenia triggered by these mAbs. A similar phagocytosis enhancement after binding with IgM antibodies, and sometimes without complement involvement, has previously been reported for apoptotic cells and infectious agents.[20–22]

Could CBA mouse immunization with rat platelets serve as a model for immune thrombocytopenic purpura (ITP) or for other human antiplatelet autoimmune response such as post-transfusion purpura? Similarities exist, since autoantibodies in ITP patients bind the same antigens as those likely recognized by our mouse antiplatelet mAbs.[23] Moreover, our data point towards several different questions that require additional investigation. First, in addition to IgG autoantibodies, human IgM antiplatelet autoantibodies are pathogenic,[24] suggesting that our mouse IgM autoantibodies can be used for pathogenicity studies. In particular, phagocytosis of platelets opsonized by IgM autoreactive antibodies may be analyzed with these mAbs, which has not yet been extensively done with this autoantibody isotype. Second, the preferential binding of activated platelets might explain the disproportionate pathogenic effect of autoantibodies that induce only moderate thrombocytopenia. Third, it will be interesting to determine the role of platelet function impairment, in addition to enhanced clearance of opsonized platelets, in the pathogenicity of antiplatelet autoantibody-mediated diseases. In this respect, the distinct impairment of platelet functions obtained with the two mAbs might suggest that a broad specificity of the autoantibody response could have more pathogenic consequences than a more restricted production. Finally, in addition to their pathogenicity, the immune mechanisms leading to the production of these autoantibodies in mice immunized with rat platelets, such as potential involvement of autoreactive T lymphocytes, are yet to be determined.

Author contributions: All authors participated in the interpretation of the studies and review of the manuscript; LD and DS conducted the experiments; NvR supplied critical reagents (liposomes); LD and J-PC wrote the manuscript, and participated in the design of the study.

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