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Brief report

PF4/heparin complexes are T cell–dependent antigens

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Heparin-induced thrombocytopenia (HIT) is a life-threatening, thrombotic disorder associated with development of anti–platelet factor 4 (anti-PF4)/heparin autoantibodies. Little is known about the antigenic and cellular requirements that initiate the immune response to these complexes. To begin to delineate mechanisms of autoantibody formation in HIT, we studied the immunizing effects of murine PF4 (mPF4)/heparin in mice with and without thymic function. Euthymic mice were injected with mPF4/heparin complexes, mPF4, heparin, or buffer. Mice injected with mPF4/heparin, but not mPF4 or heparin alone, developed heparin-dependent autoantibodies that shared serologic and functional characteristics of human HIT antibodies, including preferential binding to mPF4/heparin complexes and causing heparin- and FcRγIIA-dependent platelet activation. In contrast, athymic mice did not develop HIT-like antibodies. Taken together, these studies establish that PF4/heparin complexes are highly immunogenic and elicit self-reacting anti-PF4/heparin antibodies in a T cell–dependent manner. (Blood. 2005;106:929-931)

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Introduction

Heparin-induced thrombocytopenia (HIT) is a commonly acquired immune disorder caused by autoantibodies to complexes between platelet factor 4 (PF4) and heparin. We have previously shown in a mouse model that passive infusion of PF4/heparin antibodies into transgenic mice expressing human PF4 and FcγRIIA triggers the salient features of the human disease, thrombocytopenia and thrombosis.[1]

However, little is known about the initiation of the HIT immune response. In particular, the role of T cells in this disorder remains to be defined, because HIT has characteristics of both T cell–dependent (TD) and T cell–independent (TI) responses. On the one hand, serologic studies consistently demonstrate the importance of isotype switching,[2] a process that often requires CD4 T-cell help, and clonal restriction in the T-cell repertoire of patients with HIT has been reported.[3,4] On the other hand, HIT has features that are atypical for a TD immune disorder. Whereas TD drug reactions to drugs like penicillin or sulfonamides[5,6] are typically long-lived and associated with immunologic memory, antibodies to PF4/heparin appear to be transient[7] and recurrences do not invariably follow heparin reexposure, suggesting the absence of immunologic memory in HIT.[7,8] These observations, in addition to the extraordinary prevalence of PF4/heparin antibody formation in clinical settings such as cardiopulmonary bypass,[9] cast doubt on the requirement for T-cell help in the generation of anti-PF4/heparin antibodies.

Therefore, to begin to delineate the relevant antigenic and cellular mechanisms that lead to PF4/heparin antibody production in vivo, we studied the sensitizing effects of PF4 and heparin in mice that have or lack thymic function. Our studies indicate that heparin is antigenic only in the presence of PF4 and that PF4/heparin antibody production in vivo is dependent on thymic function.

Study design

Murine immunization model

Eight- to 10-week-old mice (BALB/c, Jackson Laboratory, Bar Harbor, ME; and BIG:BALB/c-Nu, Cancer Research Isolation Facility, Duke University Medical Center, Durham, NC) were immunized intravenously daily for 5 days via the tail vein or using retro-orbital injection. Sterile solutions containing murine (m) PF4 (20 μg per mouse) and/or heparin (0.4 units per mouse, Heplock; Elkins-Sinn, Cherry Hill, NJ) or dinitrophenol (DNP)–Ficoll (50 μg per mouse; Biosearch Technologies, Novato, CA) were prepared in Hanks balanced salt solution in a final volume of 50 μL. Blood for enzyme-linked immunosorbent assay (ELISA) was collected from the retro-orbital plexus of anesthetized mice in 3.2% sodium citrate. All studies were performed with the approval of the Institutional Animal Care & Use Committee at Duke University.

mPF4 expression, mPF4/heparin ELISA, and assays of platelet activation

mPF4 was expressed and isolated from an *Escherichia coli* expression vector as previously described.[10] Isolated mPF4 protein ran as a single band at a molecular weight (Mw) of about 7 to 8 kDa and was immunoreactive with a polyclonal anti–human PF4 (hPF4) antibody developed in our laboratory (data not shown). Isolated protein was used for injections as well as for developing an mPF4/heparin ELISA using protocols similar

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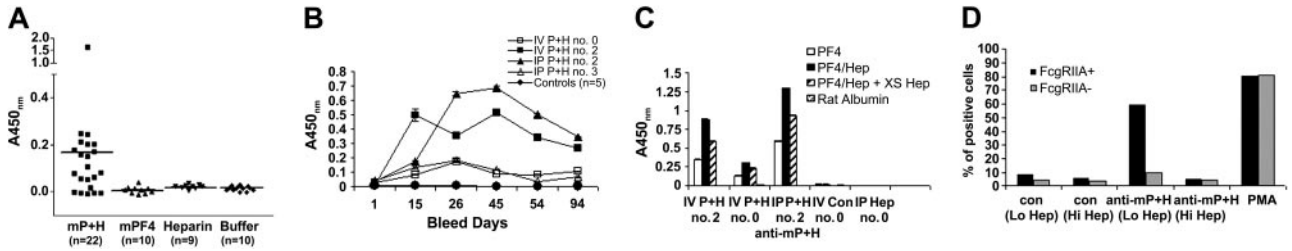

Figure 1. Characterization of a murine immunization model of anti-PF4/heparin. (A) Antibody formation. Euthymic BALB/c mice were injected intravenously with antigens (mP+H, mPF4, heparin, or buffer) as described in "Study design." Antibodies to the immunizing antigen were measured by ELISA 14 days later. Horizontal bars indicate the mean. (B) Temporal course of antibody formation. Mice injected as with mP+H (by intravenous or intraperitoneal route) or buffer (controls) were followed for about 90 days from the onset of immunization. Error bars indicate standard deviation. (C) Antigen specificity of anti-mP+H. Mice developing high-titer antibody responses after intravenous or intraperitoneal injection (IV P+H no. 2, IV P+H no. 0, and IP P+H no. 2) were tested for antigen specificity toward mPF4, mPF4/heparin, mPF4/heparin in the presence of excess heparin and to rat albumin. (D) Heparin-dependent platelet activation by anti-mPF4/heparin antibody. Murine plasma containing PF4/heparin autoantibody (anti-mP+H) or control plasma (con) in the presence of low-dose (0.02 U/mL; Lo Hep) or high-dose heparin (20 U/mL; Hi Hep) was incubated with WT platelets lacking FcγRIIA or platelets from transgenic mice expressing human FcγRIIA (FcγRIIA$^+$). Phorbol 12-myristate 13-acetate (PMA) was used as a positive control. Platelet activation was measured by expression of annexin V binding as described in "Study design."

to that described by us for anti–human PF4/heparin.[11] Flow cytometry to detect heparin-dependent platelet activation was performed as previously described.[12]

Statistical analysis

Antibody responses were compared using the Student *t* test for comparisons of 2 groups or analysis of variance (ANOVA) for 3 or more groups. Statistical analyses were performed using Graph Pad Prism (Graphpad Software, San Diego, CA). Differences were considered significant at P less than .05.

Results and discussion

These studies were undertaken to examine the mechanism underlying autoantibody formation in HIT. Mice injected with intravenous mPF4/heparin developed significantly higher levels of anti-mPF4/heparin antibody than mice injected with heparin alone, mPF4 alone, or buffer (Figure 1A; mean $A_{450nm} \pm$ SD: mPF4/heparin, 0.174 ± 0.336; mPF4, 0.008 ± 0.015; heparin, 0.022 ± 0.009; and buffer, 0.015 ± 0.009; $P < .022$ by ANOVA with Kruskal-Wallis test). These findings support the hypothesis that heparin becomes antigenic only upon binding to PF4 and suggest that it is reasonable to attribute sensitization to heparin to the formation of PF4/heparin complexes.

In humans, sensitization to PF4 may begin within the vasculature. PF4, released by activated platelets,[13] binds reversibly to glycosaminoglycans (GAGs) expressed on endothelial cells[14] and is displaced by infused heparin.[15] There is a 15- to 300-fold increase in the plasma concentration of PF4 in disease states associated with acute or chronic platelet activation, such as pulmonary embolism, atherosclerosis, or cardiopulmonary bypass.[16-18] We recently showed that the vascular burden of PF4 increases during the progression of atherosclerosis,[19] a clinical setting in which PF4/heparin autoantibodies commonly develop. Further, the knowledge that PF4 forms antigenic complexes with GAGs on monocyte/macrophages[20,21] suggests that similar events are likely to occur on dendritic cells within the immune compartment, thus "priming" the immune system in susceptible individuals.

We suspect that the absence of vascular disease or other causes of persistent platelet activation in mice, which primes the immune response in humans, explains both the requirement for exogenous mPF4 and for the delay in antibody formation. Although mice immunized with mPF4/heparin develop antibodies within 14 days, peak responses were not seen until 3 to 4 weeks after immunization (Figure 1B). Antibody titers (concentrations) in our mice varied considerably, similar to human HIT (Figure 1B). The basis for this heterogeneity in antibody development is unknown but is likely due to genetic variation that may resolve when more homogenous cohorts are developed following extensive backcrossing.

We also observed that murine anti-PF4/heparin antibodies shared important serologic and functional characteristics with HIT autoantibodies. First, murine anti-PF4/heparin antibodies were predominantly of the immunoglobulin G_1(IgG_1) subclass (data not shown), as are HIT antibodies.[22,23] Second, murine antibodies showed greater binding to mPF4/heparin than to mPF4 alone, and binding was inhibited by excess heparin (Figure 1C). Third, murine antibodies activated platelets in a heparin-dependent manner (Figure 1D). Fourth, like HIT antibodies,[24] platelet activation by murine antibodies was dependent on the expression of platelet FcγRIIA, as murine antibodies activated platelets transgenic for human FcγRIIA (FcγRIIA$^+$),[1] but not those from wild-type (WT) mice that lack a homologous receptor. Lastly, murine anti-PF4/heparin activated FcγRIIA$^+$-expressing murine platelets in the presence of low concentrations of heparin, and activation was inhibited at higher heparin concentrations (Figure 1D), which recapitulates another characteristic behavior of HIT antibodies.

Once we demonstrated that murine anti-PF4/heparin shared biologically relevant properties with HIT antibodies, we examined the cellular requirements for antibody production in vivo. To

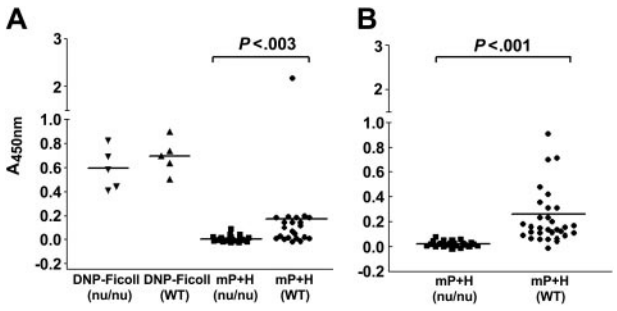

Figure 2. T-cell requirement for anti-mPF4/heparin antibody development. (A) ELISA measurements (A_{450nm}) of antibody development 14 days after inoculation of either mPF4/heparin (mP+H) or DNP-Ficoll into either euthymic BALB/c WT mice or BALB/c athymic (nu/nu) mice. Individual measurements are shown as well as mean (bar). Statistically different values are indicated at the top. (B) Antibody measurements of nu/nu and WT mice injected with mP+H 42 days after inoculation.

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investigate the role of T cells in the immune response to PF4/heparin, we immunized mice lacking thymic function (nu/nu mice) with mPF4/heparin (n = 23) or DNP-Ficoll (n = 5), a TI antigen. Antibody responses in athymic mice injected with mPF4/heparin were negligible compared with WT BALB/c mice injected using the same protocol whether assayed at 14 days (Figure 2A; mean $A_{450nm} \pm$ SD: nu/nu, 0.003 ± 0.025; versus WT, 0.175 ± 0.459; $P < .003$ by Mann-Whitney) or 42 days (Figure 2B; $A_{450nm} \pm$ SD: nu/nu, 0.02 ± 0.023; versus WT, 0.262 ± 0.303; $P < .001$ by Mann-Whitney). This contrasts with the immune response of athymic and WT mice injected with DNP-Ficoll, wherein antibody responses were comparable (Figure 2A; mean $A_{450nm} \pm$ SD: nu/nu, 0.589 ± 0.173; versus WT, 0.694 ± 0.146; $P =$ not significant [ns]).

In conclusion, our murine studies indicate that PF4/heparin complexes are immunogenic in vivo in contrast to PF4 or heparin alone. These autoantibodies have similar characteristics to HIT antibodies. Most importantly, we show in our murine system that the production of these anti-PF4/heparin antibodies requires T cells that have been educated in the thymus. This mouse model of autoantibody production will enable future studies to delineate the immunoregulatory and pathogenic mechanisms involved in the development of HIT.

References

1. Reilly MP, Taylor SM, Hartman NK, et al. Heparin-induced thrombocytopenia/thrombosis in a transgenic mouse model requires human platelet factor 4 and platelet activation through FcγRIIA. Blood. 2001;98:2442-2447.
2. Denomme GA, Warkentin TE, Horsewood P, Sheppard JA, Warner MN, Kelton JG. Activation of platelets by sera containing IgG1 heparin-dependent antibodies: an explanation for the predominance of the Fc γRIIa "low responder" (his131) gene in patients with heparin-induced thrombocytopenia . J Lab Clin Med. 1997;130: 278-284.
3. Bacsi S, De Palma R, Visentin GP, Gorski J, Aster RH. Complexes of heparin and platelet factor 4 specifically stimulate T cells from patients with heparin-induced thrombocytopenia/thrombosis. Blood. 1999;94:208-215.
4. Bacsi S, Geoffrey R, Visentin G, De Palma R, Aster R, Gorski J. Identification of T cells responding to a self-protein modified by an external agent. Hum Immunol. 2001;62:113-124.
5. Depta JP, Pichler WJ. Cross-reactivity with drugs at the T cell level. Curr Opin Allergy Clin Immunol. 2003;3:261-267.
6. Ring J, Brockow K. Adverse drug reactions: mechanisms and assessment. Eur Surg Res. 2002;34:170-175.
7. Warkentin TE, Kelton JG. Temporal aspects of heparin-induced thrombocytopenia. N Engl J Med. 2001;344:1286-1292.
8. Potzsch B, Klovekorn WP, Madlener K. Use of heparin during cardiopulmonary bypass in patients with a history of heparin-induced thrombocytopenia [letter]. N Engl J Med. 2000;343:515.
9. Warkentin TE, Sheppard JA, Horsewood P, Simpson PJ, Moore JC, Kelton JG. Impact of the patient population on the risk for heparin-induced thrombocytopenia. Blood. 2000;96:1703-1708.
10. Li ZQ, Liu W, Park KS, et al. Defining a second epitope for heparin-induced thrombocytopenia/thrombosis antibodies using KKO, a murine HIT-like monoclonal antibody. Blood. 2002;99:1230-1236.
11. Arepally G, Reynolds C, Tomaski A, et al. Comparison of PF4/heparin ELISA assay with the ^{14}C-serotonin release assay in the diagnosis of heparin-induced thrombocytopenia. Am J Clin Pathol. 1995;104:648-654.
12. Rauova L, Poncz M, McKenzie SE, et al. Ultra-large complexes of PF4 and heparin are central to the pathogenesis of heparin-induced thrombocytopenia. Blood. 2005;105:131-138.
13. Zucker MB, Katz IR. Platelet factor 4: production, structure, and physiologic and immunologic action. Proc Soc Exp Biol Med. 1991;198:693-702.
14. Stern D, Nawroth P, Marcum J, et al. Interaction of antithrombin III with bovine aortic segments. Role of heparin in binding and enhanced anticoagulant activity. J Clin Invest. 1985;75:272-279.
15. Rao AK, Niewiarowski S, James P, et al. Effect of heparin on the in vivo release and clearance of human platelet factor 4. Blood. 1983;61:1208-1214.
16. Handin RI, McDonough M, Lesch M. Elevation of platelet factor four in acute myocardial infarction: measurement by radioimmunoassay. J Lab Clin Med. 1978;91:340-349.
17. Cella G, Colby SI, Taylor AD, McCracken L, Parisi AF, Sasahara AA. Platelet factor 4 (PF4) and heparin-released platelet factor 4 (HR-PF4) in patients with cardiovascular disorders. Thromb Res. 1983;29:499-509.
18. Cella G, Vittadello O, Gallucci V, Girolami A. The release of beta-thromboglobulin and platelet factor 4 during extracorporeal circulation for open heart surgery. Eur J Clin Invest. 1981;11:165-169.
19. Pitsilos S, Hunt J, Mohler ER, et al. Platelet factor 4 localization in carotid atherosclerotic plaques: correlation with clinical parameters. Thromb Haemost. 2003;90:1112-1120.
20. Pouplard C, Iochmann S, Renard M, et al. Induction of monocyte tissue factor expression by antibodies to platelet factor 4 developed in heparin-induced thrombocytopenia [abstract]. Blood. 2000;96:530a.
21. Arepally GM, Mayer IM. Antibodies from patients with heparin-induced thrombocytopenia stimulate monocytic cells to express tissue factor and secrete interleukin-8. Blood. 2001;98:1252-1254.
22. Arepally G, McKenzie SE, Jiang XM, Poncz M, Cines DB. FcγRIIA H/R 131 polymorphism, subclass-specific IgG anti-heparin/platelet factor 4 antibodies and clinical course in patients with heparin-induced thrombocytopenia and thrombosis. Blood. 1997;89:370-375.
23. Suh JS, Malik MI, Aster RH, Visentin GP. Characterization of the humoral immune response in heparin-induced thrombocytopenia. Am J Hematol. 1997;54:196-201.
24. Chong BH, Castaldi PA, Berndt MC. Heparin-induced thrombocytopenia: effects of rabbit IgG, and its Fab and FC fragments on antibody-heparin-platelet interaction. Thromb Res. 1989;55: 291-295.

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