Clin Exp Immunol 1997; **108**:333–339

Pathogenicity of human anti-platelet factor 4 (PF4)/heparin *in vivo*: generation of mouse anti-PF4/heparin and induction of thrombocytopenia by heparin

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(Accepted for publication 13 January 1997)

SUMMARY

Heparin-induced thrombocytopenia/thrombosis (HIT) is a severe thrombotic disorder that occurs in ≈1% of patients treated with heparin. Affected patients commonly develop antibodies that recognize PF4/heparin complexes that may form on the surface of activated platelets and on the endothelium. However, it has not been established that anti-PF4/heparin antibodies are responsible for the clinical manifestations of HIT. To address this issue, we employed a recently developed model of active immunity to study the effect of IgG anti-PF4/heparin antibody *in vivo*. In previous studies we have shown that it is possible to induce autoimmune diseases such as systemic lupus erythematosus (SLE), anti-phospholipid syndrome (APS) or vasculitis in naive mice by active immunization with anti-DNA, anti-cardiolipin and anti-neutrophil cytoplasmic antibodies, respectively. Immunized animals develop anti-idiotypic antibodies (Ab_2) and, after 2–4 months, anti-anti-idiotypic antibodies (Ab_3). Ab_3s generated in this manner often simulate the binding activity of Ab_1 and their expression correlates with the development of specific clinical manifestations typical of the respective human disease. Based on this experience, naive BALB/c mice were immunized with IgG anti-PF4/heparin antibodies isolated from two patients with HIT. The actively immunized mice developed mouse anti-PF4/heparin antibody (Ab_3). Administration of unfractionated heparin, but not low molecular weight heparin (LMWH), to the actively immunized animals induced thrombocytopenia by day 4 of drug exposure. There was no evidence of thrombosis. The results of this study support the importance of anti-PF4/heparin antibodies in the pathogenesis of HIT**.** Further, this model may help to elucidate the factors responsible for thrombosis as well as providing means to assess new treatment options for patients with this disorder.

Keywords Heparin-induced thrombocytopenia anti-PF4/heparin low molecular weight heparin anti-cardiolipin anti-endothelial cell antibody thrombocytopenia

INTRODUCTION

Heparin is the most frequent cause of drug-induced immune thrombocytopenia. Paradoxically, affected patients commonly present with thromboembolic phenomena [1–4]. Indeed, the frequent development of recurrent arterial and/or venous thrombosis in patients with heparin-induced thrombocytopenia (HIT) is almost unique among drug-induced immune platelet disorders.

It is now widely accepted that the thrombocytopenia in HIT is caused by antibodies that bind to platelets in the presence of heparin [4–6]. Recently, it has been found that at least some of these antibodies bind to complexes between oligosaccharides such as heparin [7–11] and PF4, a tetrameric peptide secreted by activated platelets [12–14] or, less commonly, other heparin-binding proteins [15]. PF4 binds to the surface of activated platelets, where it is presumed to become a target for HIT antibodies, thereby promoting platelet clearance [12–16].

It is less clear how anti-PF4/heparin antibodies cause thrombosis**.** Most investigators have emphasized the possibility that platelets become activated as a result of secondary interactions between platelet-bound IgG PF4/heparin complexes and FcγRIIA receptors expressed on neighbouring platelets [11,17–20]. However, it has not yet been established that cross-linking these receptors occurs *in vivo* or is unique to HIT. Others have invoked the possibility that HIT antibodies recognize PF4 bound to heparin or proteoglycans containing heparin-like sequences expressed by endothelial cells [7,17]. It has also been proposed that monocytes exposed to PF4 and HIT antibody may contribute to thrombosis

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through increased expression of P selectin and tissue factor [17,21].

To gain insight into the pathogenesis of HIT and the role of anti-PF4/heparin antibodies, we took advantage of a recently developed murine model of active immunization. Previously, we [22–27] and others [28–30] have shown that it is possible to induce autoimmune conditions by dysregulating the idiotypic network [22–30] through active immunization with a pathogenic autoantibody (i.e. idiotype). Upon stimulation with the autoantibody carrying a specific idiotype (Ab_1), naive mice develop anti-autoantibodies (anti-Id = Ab_2), and after 1–2 months**,** anti-anti-autoantibodies (anti-anti-Id = Ab_3) that may have similar binding specificities to Ab_1. Coincident with expression of Ab_3, immunized mice often develop an overt autoimmune condition that resembles the human disorder from which the inducing autoantibody (Ab_1) was obtained, e.g. systemic lupus erythematosus (SLE) in mice immunized with anti-DNA [22–28], antiphospholipid syndrome (APS) in mice immunized with anti-cardiolipin [24–26], and vasculitis in mice immunized with classical anti-neutrophil cytoplasmic antibody (C-ANCA) [31,32]. This method may be used to elicit an autoimmune phenomenon which may not be recapitulated through passive immunization of the autoantibody (Ab_1). As well, this murine model of disease makes possible the study of an autologous antibody (i.e. Ab_3, a mouse autoantibody) *in vivo*, rather than a limited characterization of a heterologous antibody (i.e. a human autoantibody) in passive transfer experiments.

Based on this experience, we employed a similar approach to study the putative pathogenic role of anti-PF4/antibodies. The results of the present study indicate that naive mice immunized with IgG anti-PF4/heparin antibody from patients with HIT produced Ab_3, i.e. murine anti-PF4/heparin, and developed thrombocytopenia when exposed to unfractionated, but not to low molecular weight heparin (LMWH).

MATERIALS AND METHODS

Human antibodies

Plasma from two patients with a clinical [33] and serologic [33,34] diagnosis of HIT were studied. Plasma from each patient caused ^{14}C-serotonin release from healthy donor platelets only in the presence of added heparin (< 0·5 U/ml) and contained antibodies to PF4/heparin complexes [34]. One patient (HIT-1) presented with a deep venous thrombosis at the time HIT was discovered; the other patient developed asymptomatic thrombocytopenia. IgGs from both patients and from a subject with no history of heparin exposure were isolated using staphylococcus protein A agarose (Pharmacia Biotech, Uppsala, Sweden). The IgG migrated as two bands at mol. wt ≈50 kD and ≈27 kD on SDS–PAGE under reducing conditions. No high mol. wt complexes were detected either on SDS–PAGE or on gel filtration using reverse phase fast performance liquid chromatography (FPLC). All IgG fractions were depleted of cardiolipin (CL) or phosphatidylserine (PS) reactivity using silica beads precoated with each phospholipid, as previously described [24,30]. IgG samples depleted of CL or PS reactivity were further tested for trace amounts of phospholipids using an anti-phospholipid MoAb in an ELISA.

Induction of mouse anti-PF4/heparin and thrombocytopenia

Mouse anti-PF4/heparin antibodies were induced using protocols previously described by us to induce other autoantibodies and autoimmune disorders in naive mice [22–28,31,32]. Briefly, 14-week-old BALB/c mice were immunized intradermally in the hind footpads with each IgG preparation (20 μg emulsified in Freund's complete adjuvant); 15 mice per group were immunized with IgG from either patient (HIT-1 or HIT-2) or with control IgG**.** Three weeks later, a booster injection of the same antibodies in PBS was given in the same manner. When the mice were found to have produced anti-PF4/heparin antibodies (≈ 2 months after the booster injection), they were given unfractionated heparin (Leo Pharmaceutical Products, Ballerup, Denmark; Lot F44 A Exp. 97) subcutaneously, 5 U/mouse daily for 4 days (a dose equivalent to 10 000 U twice a day in humans). Other mice received Clexane, a LMWH (May and Baker, Paris, France), 0·02 mg/mouse subcutaneously twice a day (equivalent to 40 mg twice a day in humans). The platelet count was determined daily for the next 10 days using a single optical cytometer (HC Plus Cell Control; Coulter Electronics Ltd, Luton, UK). Antibody titres were studied episodically over a period of 5 months until the animals were killed to look for thrombosis.

Detection of mouse anti-human anti-PF4/heparin (Ab_2)

ELISA 96-well microplates (Nunc, Roskilde, Denmark) were coated with affinity-purified F(ab′)$_2$ human anti-PF4/heparin or irrelevant human IgG (2 μg/ml) in $NaHCO_3$ 0·5 M pH 9·5, and incubated at 4°C overnight. After washing, plates were blocked with 1% bovine serum albumin (BSA) in PBS for 2 h at room temperature. Murine sera were added at a dilution of 1 : 200. Binding of mouse anti-human PF4/heparin (Ab_2) was probed by adding goat anti-mouse Fc, conjugated to alkaline phosphatase (Jackson Immunoresearch Labs Inc., West Grove, PA). *p*-nitrophenyl phosphate (Sigma Chemical Co., St Louis, MO) was added, and absorbance at 405 nm was measured (SLT-Labinstruments, Vienna, Austria).

Affinity purification of mouse anti-anti-human anti-PF4/heparin (Ab_3, or mouse anti-PF4/heparin)

IgG was isolated from pooled mouse sera using anti-mouse IgG coupled to CNBr-activated Sepharose (Pharmacia). A 5-ml column of heparin-Sepharose CL-6B (Pharmacia) was incubated with human PF4 (Sigma). Murine IgG was added and the mixture was incubated overnight at 4°C with shaking. The heparin/PF4 column was extensively washed with PBS and 2-ml fractions were collected, as previously described [35]. Bound IgG was eluted stepwise with 20 ml each of 0·3 M, 0·6 M, 1 M, 3 M NaCl and 4 M guanidine HCl/NaCl 2 M, pH 7·4. The peak fractions were dialysed against PBS. Binding of immunoglobulin eluted from the heparin/PF4 column to heparin, PF4, PF4/heparin and fibronectin/heparin was tested by ELISA.

Detection of mouse anti-PF4/heparin antibodies (Ab_3) by ELISA

Murine anti-PF4/heparin antibodies were detected using an ELISA based on the method described by Amiral *et al.* [13] and Shibata *et al.* [35]. Briefly, polystyrene microtitre plates (Nunc) were coated with 10 μg/ml of protamine sulfate in 0·1 M sodium carbonate pH 9·8 for 1 h at room temperature. Protamine sulfate-coated plates were washed with PBS–0·1% Brij (PBSB) and then incubated overnight at 4°C with 25 μg/ml human PF4 alone, 0·2 U/ml unfractionated heparin alone, 0·2 U/ml unfractionated heparin with 25 μg/ml human PF4 (Sigma) or 25 μg/ml human fibronectin with 0·2 U/ml unfractionated heparin in PBS. Plates were washed three times with PBSB and unreactive sites were blocked for 1 h with 3% BSA–PBS at room temperature. Sera from immunized mice (1 : 5–1 : 1000) and subsequently, affinity-purified mouse anti-human

PF4 IgG (Ab_3) in 1% BSA–PBS were added to plates for 2 h at room temperature. After three washes with PBSB, bound IgG was detected by adding goat anti-mouse IgG alkaline phosphatase (Jackson) in 1% BSA–PBS for 1 h at room temperature, the plates washed again, the appropriate substrate added and the optical density (OD) at 405 nm was measured. Reactions were considered to be positive when the mean OD obtained with PF4/heparin complexes was at least 2 s.d. above the value obtained with PF4 or heparin alone, or with plates coated with heparin/fibronectin complexes (fibronectin was supplied by Sigma).

Inhibition studies

The specificity of murine affinity-purified anti-PF4/heparin IgG (Ab_3) binding to various immobilized antigens was examined by competitive inhibition. IgG, diluted to give 50% of its maximal binding to PF4/heparin, was preincubated with PF4/heparin (0·8 U heparin/100 μg PF4, 0·4 U heparin/50 μg PF4, 0·2 U heparin/25 μg PF4, etc.). Residual anti-PF4/heparin activity was determined by ELISA as described above.

Competition studies were also performed of human and mouse anti-PF4/heparin immunoglobulins (Ab_1 *versus* Ab_3) to characterize the binding specificities of the two antibodies. Human anti-PF4/heparin IgG (Ab_1), diluted to give 50% of maximal binding to PF4/heparin, was preincubated with different concentrations of mouse anti-PF4/heparin (Ab_3). Normal human IgG was also used instead of Ab_1 in parallel fashion to serve as control. Residual anti-PF4/heparin activity was determined by ELISA employing an anti-human IgG conjugated to alkaline phosphatase as described above.

Percent inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{OD control} - \text{OD with competitor}}{\text{OD control}} \times 100$$

Detection of anti-endothelial cell antibodies (AECA)

Binding of the mouse sera or purified IgG to murine endothelial cells was tested by cyto-ELISA [36]. Ninety-six-well tissue culture plates (Nunc) were seeded with H5V cells (mouse endothelioma cells originated from C57Bl/6 mice, kindly provided by Dr P. L. Meroni, Milan, Italy) in Dulbecco's minimal essential medium (DMEM)-enriched media supplemented with 10% fetal calf serum (FCS), 3% glutamine, 1% HEPES buffer, 1% non-essential amino acids, 1% sodium pyruvate, 1% streptomycin-penicillin overnight at 4°C. Unreactive sites were blocked with 0·5% BSA in Hanks' media for 30 min at 37°C and washed extensively. In order to clarify whether the anti-PF4/heparin-purified IgG binds directly to endothelial cells (EC) or requires the presence of PF4 for binding to EC, 100 μg/ml PF4 (Sigma) were added to half the EC cultures for 2 h at 37°C. As irrelevant antigen, β_2-glycoprotein I at 10 μg/well (β_2-GPI; kindly donated by Professor Angela Tincani, Brescia, Italy) was added in a similar manner. β_2-GPI was chosen because it has been reported to serve as a bridge for binding of anti-cardiolipin antibodies to endothelial cells as well as to cardiolipin [36–39]. Mouse sera or purified IgG (1–100 μg/ml in Hanks'/0·5% BSA) were added for 2 h at room temperature. The wells were washed to remove unbound immunoglobulin, and peroxidase-conjugated anti-mouse IgG (Jackson Immunoresearch Labs) was added for 1 h at room temperature in Hanks'. *o*-phenylenediamine dihydrochloride (OPD; Sigma) and H_2O_2 were added and the OD at 492 nm was measured.

Statistical analysis

One way analysis of variance (ANOVA) was used to compare results between groups. Significance was defined as $P < 0{\cdot}05$. Significance of differences between groups was determined using the Wilkinson test and Bonferoni tests.

RESULTS

Induction of murine anti-PF4/heparin antibodies (Ab_3)

The goal of our studies was to develop a murine model of HIT. To do this, we studied the antibody response of naive BALB/c mice injected with affinity-purified IgG containing anti-PF4/heparin antibodies (Ab_1) from two patients with HIT. These affinity-purified human antibodies bound specifically to PF4/heparin, and not to PF4/LMWH, fibronectin/heparin, PF4 alone or heparin alone (Fig. 1). All immunized mice (15 per group), developed specific murine anti-human PF4/heparin antibodies (Ab_2), as shown in Fig. 2. The titres of Ab_2 decreased 8–12 weeks after the first injection of human anti-PF4/heparin antibody.

Mouse anti-PF4/heparin (Ab_3) developed within 1–2 months after the initial and a single booster immunization. The specificity of this antibody development was shown in several ways. First, Ab_3 did not bind to heparin or to complexes composed of heparin and another heparin-binding protein, fibronectin (Fig. 3). Second, anti-PF4/heparin antibodies were not detected in mice immunized in the same manner with IgG from an individual never exposed to heparin (Figs 2 and 3). Third, binding of Ab_3 from mice immunized with HIT-1 IgG and HIT-2 IgG to immobilized PF4/heparin was inhibited in a dose-dependent manner by soluble antigen (i.e. at a dose of 12·5 μg/ml of soluble PF4/heparin inhibition of Ab_3 was 62% and 71%, respectively, but only 5–6% using the same concentrations of soluble fibronectin/heparin complexes). Finally, murine anti-PF4/heparin (Ab_3) showed competitive binding to antigen in the presence of human anti-PF4/heparin (Ab_1). Ab_3 reduced the binding of Ab_1 by 43% and 57% compared with equal concentrations of mouse IgG from control animals (Fig. 4).

The titre of mouse anti-PF4/heparin antibodies (Ab_3) reached a plateau a mean of 2 months after the booster immunization, after which it began to decline (Fig. 5).

Sera from mice producing Ab_3 deposited immunoglobulin on cultured murine endothelial cells (Fig. 6a). The specificity of this interaction was studied further using affinity-purified mouse anti-PF4/heparin (Ab_3). Ab_3 did not bind to native endothelial cells. Rather, binding of Ab_3 required the presence of exogenous PF4 (Fig. 6b) and did not occur upon the addition of comparable amounts of β_2-GPI (Fig. 6b).

Exposure of immunized mice to heparin

Two months after the booster immunization, mice were exposed to either unfractionated heparin or LMWH injected subcutaneously twice a day for 4 days. As can be seen in Fig. 7, the platelet count in mice immunized with HIT-1 IgG fell to a mean of $704 \times 10^3/\mu$l 72 h after exposure to unfractionated heparin compared with a mean platelet count of $1157 \times 10^3/\mu$l in mice immunized with control IgG ($P < 0{\cdot}001$). The platelet counts in the affected mice reached a nadir at day 4, began to rise by day 6 and reached normal levels 20 days after heparin was discontinued (not shown). The group of mice immunized with HIT-2 IgG reached a nadir platelet count 24 h after exposure to heparin ($804 \times 10^3/\mu$l); the count began to rise 2 days later and reached normal levels 3 days after heparin injections had been discontinued. None of the animals exposed to LMWH over the same time period developed thrombocytopenia. None of the mice developed macroscopically visible thrombotic lesions in

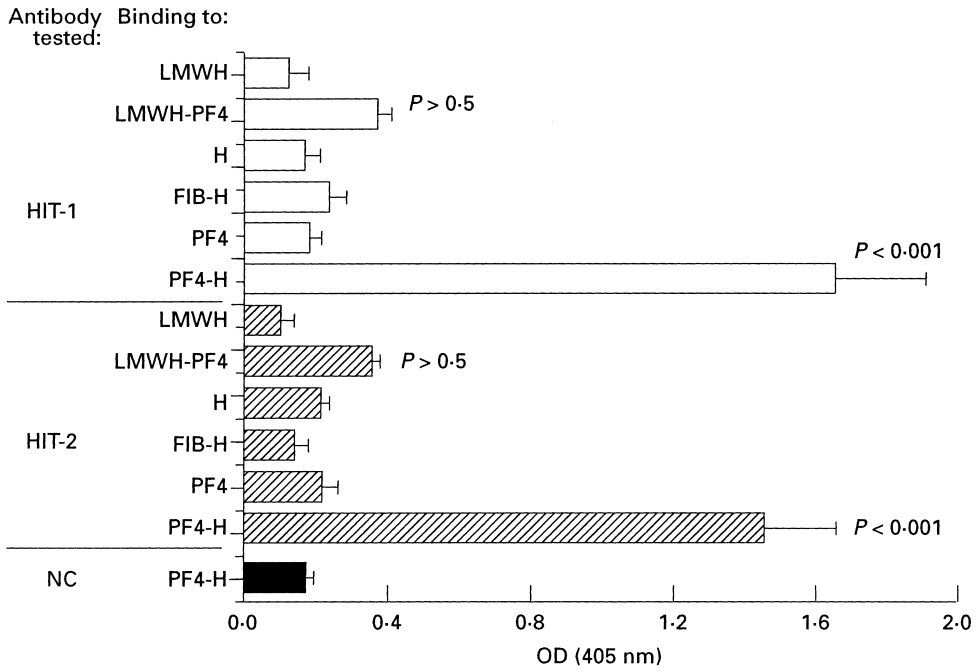

Fig. 1. Specificity of human anti-PF4/heparin binding, HIT-1 and HIT-2. IgGs purified from two patients with heparin-induced thrombocytopenia/thrombosis (HIT) and from a normal control (NC) were tested for binding to: PF4-H (PF4/heparin complex); PF4-LMWH (PF4/low molecular weight heparin complex); FIB-H (fibronectin/heparin complexes); H (unfractionated heparin); LMWH (low molecular weight heparin); PF4 (platelet factor 4). The IgGs were tested at a concentration of 10 μg/ml. Data are presented as mean + s.d. of three repeated experiments.

large and small arteries and veins in kidneys, lungs, liver and spleen of the immunized mice (e.g. employing specific stainings: Masson-trichrome and phosphattungstic acid haematoxylin).

DISCUSSION

Our studies indicate that active immunization of naive mice with human IgG anti-PF4/heparin antibodies purified from patients with HIT led to the production of murine antibodies with the same binding specificity. Further, exposure of these mice to unfractionated, but not to LMWH, induced thrombocytopenia within a matter of days.

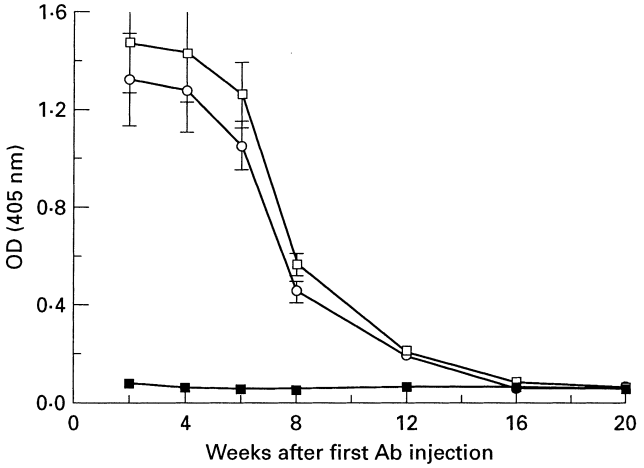

Fig. 2. Decay of mouse anti-human PF4/heparin (Ab_2). Detection of murine anti-human PF4/heparin antibody (Ab_2) titres at different time points after immunization, in mice immunized with IgG fractions of plasma from two patients with heparin-induced thrombocytopenia/thrombosis (HIT) (HIT-1 (□), HIT-2 (○)) or IgG from an individual not exposed to heparin (NC-IgG (■)).

According to Jerne's hypothesis the immune response might be regulated via antigenic determinants of immunoglobulin variable regions (idiotypes) [40,41]. The antigen (autoantigen) may stimulate the generation of Ab_1 and then the serologically unique structure of its antigen binding site (Ab_1-idiotype) stimulates the immune system to produce Ab_2. The latter recognizing the antigen binding site of Ab_1 (anti-Ab_1 or anti-Id) may mimic the Ag either conformationally or even in its amino acid sequence. If the primary antigen is itself an antibody (or an autoantibody) the specificity of Ab_3 (anti-anti-Id) may be almost identical to the original immunizing autoantibody [22,42]. The capacity of the idiotypic cascade to produce autoantibodies and, more importantly, autoimmune disease has been demonstrated in other situations [43–45]. In these settings, the similarity between Ab_1 and Ab_3 has been further confirmed by amino acid sequence analysis of the complementarity determining regions (CDRs) of their respective heavy chains [46].

By disrupting the idiotypic network of BALB/c mice through immunization with human anti-PF4/heparin antibodies (Ab_1), we successfully generated murine anti-PF4/heparin antibodies that appear to share antigen specificity with the immunizing antibody, human anti-PF4/heparin (Ab_1, Fig. 4). These antibodies are specific for PF4/heparin complexes but not for its components (Fig. 3), and they do not recognize complexes between heparin and another heparin-binding protein, fibronectin. Similar to human antibodies (Ab_1), binding of the murine anti-PF4/heparin antibodies (Ab_3) to endothelial cells requires the addition of exogenous

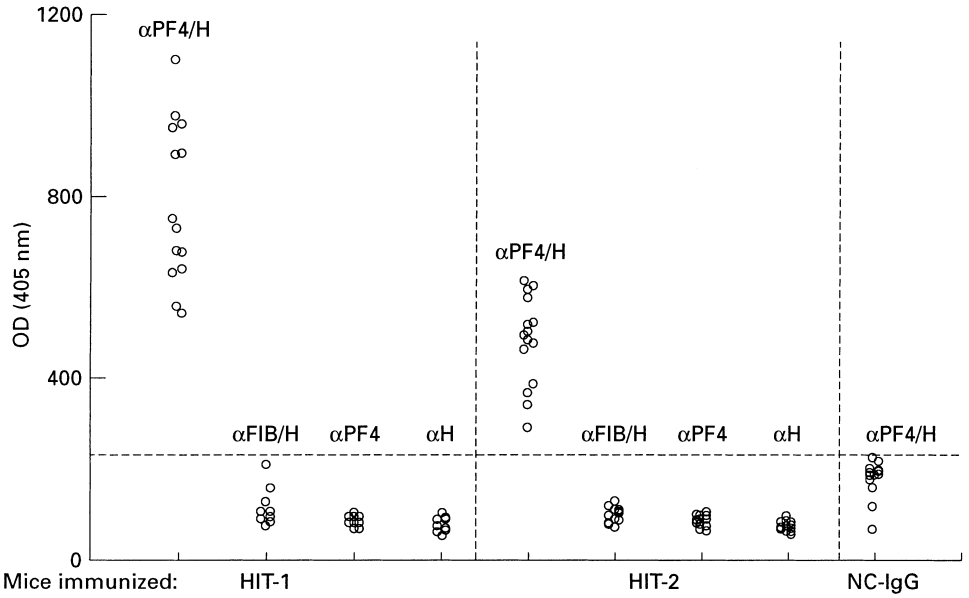

Fig. 3. Development of mouse anti-PF4/heparin antibodies (Ab_3) in immunized mice. Detection of murine anti-PF4/heparin antibodies (Ab_3) in mice immunized with IgG fractions of plasma from two patients with heparin-induced thrombocytopenia/thrombosis (HIT) (HIT-1, HIT-2) or IgG from an individual not exposed to heparin (NC-IgG). αPF4/H (antibodies to complexes between PF4 and heparin), αFIB/H (complexes between fibronectin and heparin), αPF4 (wells coated with PF4 alone), αH (wells coated with heparin alone). Murine sera were tested at a dilution of 1:5 2 months after the booster immunization. Data are expressed as the mean for individual mice (15 mice/group) tested on three separate occasions. The line (- - -) represents the mean + 2 s.d. of the activity in sera of mice injected with normal IgG.

PF4, demonstrating that the antigenic complex can be formed on this cell surface through complexation with heparan sulfate [17,47].

The *in vivo* relevance of Ab_3 was also confirmed by the fall in platelet counts in immunized mice exposed to unfractionated heparin. However, it was surprising that thrombocytopenia took several days to develop in the cohort of mice immunized with one source of HIT-IgG, since the disorder often develops in an explosive manner in sensitized human subjects. There are several potential reasons for this apparent discrepancy. First, re-exposure to heparin may be required to cause affinity maturation of the immune response over several days, ultimately yielding antibodies that have greater specificity and higher avidity for platelets. An alternative possibility is the absence of a murine analogue of FcγRIIA capable of signal transduction on murine platelets which is required to accelerate platelet–platelet interactions which, themselves, promote clearance [48–50]. This would also help to explain the seeming absence of clinically overt thromboemboli in the mice, although it should also be noted that spontaneous thromboemboli are not found in experimental models of APS [26,27]. More likely, however, the lag time to develop

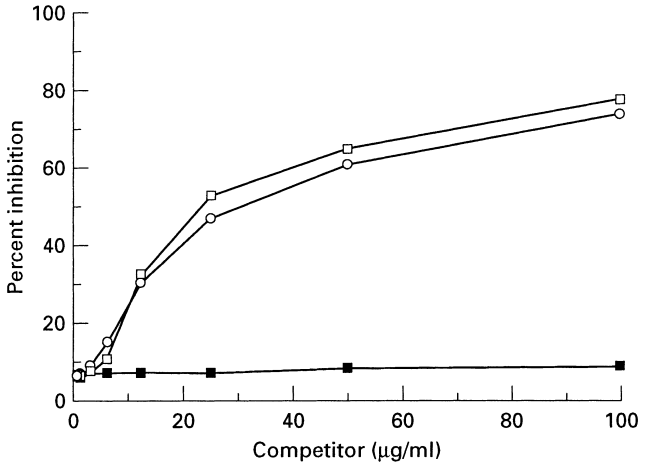

Fig. 4. Competition of mouse anti-PF4/heparin (Ab_3) with human anti-PF4/heparin (Ab_1) on binding to the antigen. Affinity-purified human IgG anti-PF4/heparin (Ab_1) was tested for specific binding to PF4/heparin in the presence of various concentrations of mouse anti-PF4/heparin (Ab_3). Competitor: mouse antiPF4/heparin from experimental heparin-induced thrombocytopenia/thrombosis (HIT). □, HIT-1; ○, HIT-2; ■, NC-IgG.

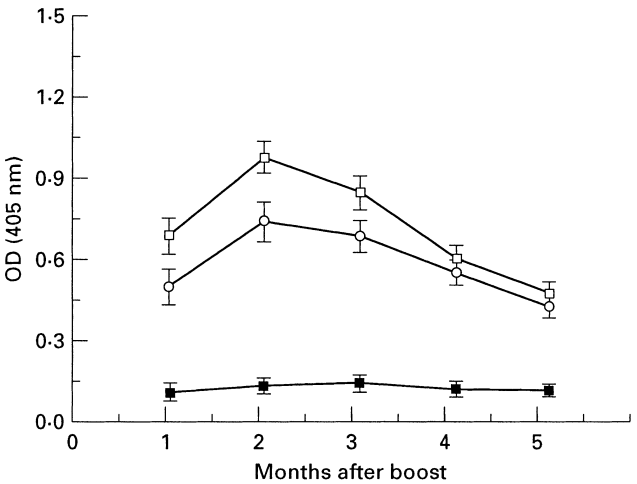

Fig. 5. Time of appearance of murine anti-PF4/heparin (Ab_3) in immunized mice. Mice were immunized with HIT-1 IgG (□), HIT-2 IgG (○) or control IgG (NC-IgG (■)) and the titres of anti-PF4/heparin were measured each month. Each point represents the mean ± 2 s.d. of sera from 15 mice.

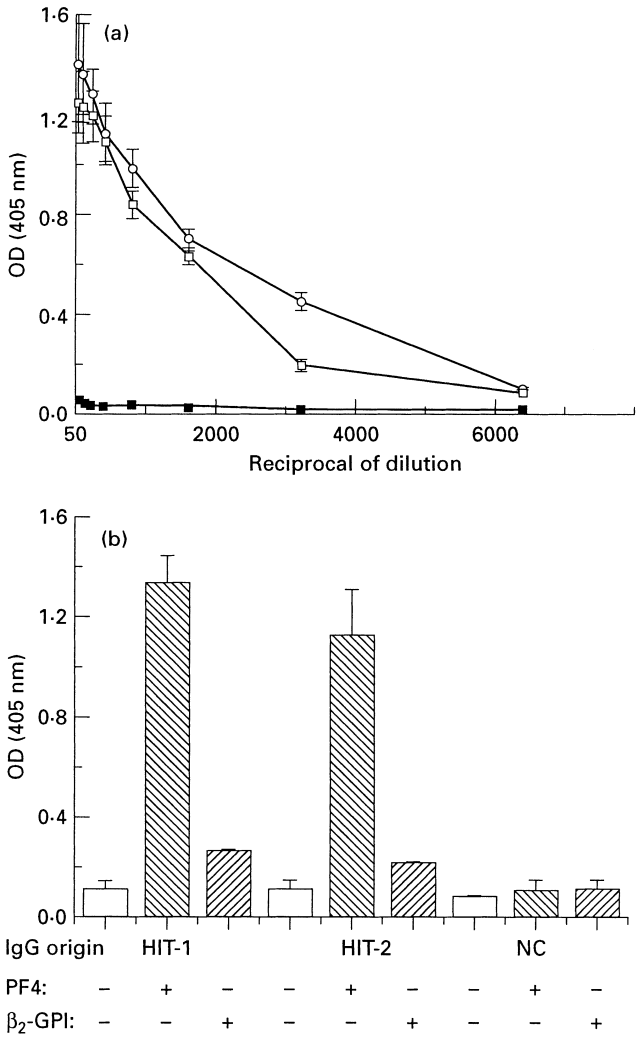

Fig. 6. (a) Anti-endothelial cell binding of sera from mice immunized with anti-PF4/heparin. Reciprocal of dilution of sera from mice immunized with IgG fractions of plasma from two patients with heparin-induced thrombocytopenia/thrombosis (HIT) (HIT-1 (□), HIT-2 (○)) or IgG from an individual not exposed to heparin (NC-IgG (■)), were tested for anti-endothelial cell binding by cytoELISA. (b) Anti-endothelial binding of affinity-purified anti-PF4/heparin. Affinity-purified anti-PF4/heparin (10 μg/ml) tested for anti-endothelial cell binding, was studied directly, in the presence of PF4 or β_2-glycoprotein I (β_2-GPI).

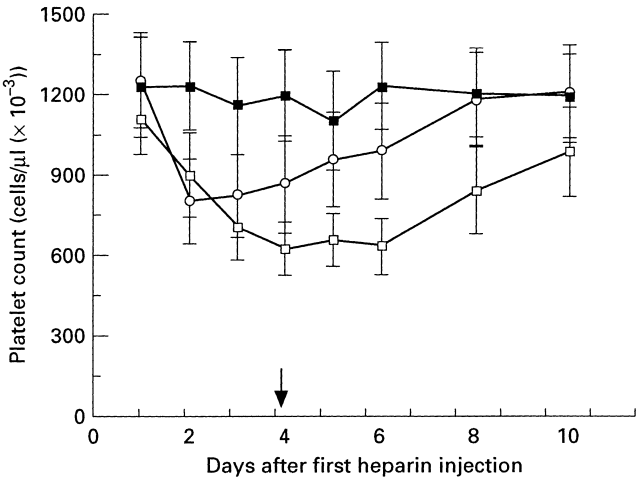

Fig. 7. Platelet counts of mice with Ab_3 upon challenge with unfractionated heparin. Platelet counts in mice immunized with HIT-1 (□) or HIT-2 (○) or NC-IgG (■) after administration of unfractionated heparin for 4 days. Each point represents the mean ± s.d. of the counts in groups of seven mice. The arrow is on day 4, when heparin injections were discontinued.

thrombocytopenia in mice as opposed to sensitized human patients reflects differences in platelet activation in the two settings, and therefore the availability of PF4 required for antibody binding to platelets. The relative paucity of PF4 in the circulation of these otherwise unmanipulated mice may also help to explain the apparent absence of thrombosis. Experiments to test the effect of endogenous and exogenous PF4 on the development of thrombocytopenia and thrombosis *in vivo* are currently underway. In addition, the capacity of affinity-purified anti-PF4/heparin antibodies to cause thrombocytopenia (and thrombosis) after transfer to naive mice subsequently exposed to heparin is currently being assessed.

Finally, it was also of interest that mice with circulating Ab_3 did not develop thrombocytopenia after exposure to LMWH. Ab_1 from both patients (HIT-1 and HIT-2) showed no cross-reactivity to PF4/LMWH complexes *in vitro*, suggesting that Ab_3 has similar biologic specificities to Ab_1 *in vivo*. Alternatively, Ab_3 may show comparable reactivity to complexes formed between PF4 and each kind of heparin *in vivo*, but thrombocytopenia in immunized mice exposed to LMWH is more likely to reflect differences in the biologic actions of the molecules. Specifically, LMWH releases less PF4 from platelets [50] and binds with lower avidity to PF4 than does unfractionated heparin [51,52], factors that may be important in this experimental model, where PF4 may be limiting. In either event, the availability of a murine model of HIT should permit detailed study of the effects of alternative forms of anticoagulation.

ACKNOWLEDGMENTS

This work was supported by a grant from the Israeli Chief Scientist-Ministry of Health (no. 3685), the Freda and Leon Schaller Grant for Research in Autoimmunity, grants from the National Institutes of Health (HL40387, HL49517, HL50790 and HL54749 to D.B.C.) and from the SEPA affiliate of the American Heart Association (G.A.).

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