Interaktionen von Thrombozytenproteinen mit Bakterien als Brücke zwischen unspezifischer und spezifischer Immunabwehr

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Inhaltsverzeichnis

1 Einleitung und Aufgabenstellung 1

2 Methoden, Ergebnisse und Diskussion 10

3 Ausblick 28

4 Abkürzungsverzeichnis 31

5 Literaturverzeichnis 34

6 Publikationen 42

6.1 A. Greinacher, K. Althaus, K. Krauel, and S. Selleng. Heparin-induced thrombocytopenia. Hämostaseologie. 2010; 30: 17-28. 42

6.2 K. Krauel, B. Fürll, T. E. Warkentin, W. Weitschies, T. Kohlmann, J. I. Sheppard, and A. Greinacher. Heparin-induced thrombocytopenia – therapeutic concentrations of danaparoid, unlike fondaparinux and direct thrombin inhibitors, inhibit formation of platelet factor 4-heparin complexes. J Thromb Haemost. 2008; 6: 2160-2167. 54

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7 Anhang 88

7.1 Zusammenfassung 88

7.2 Wissenschaftliche Leistungen 90

7.2.1 Veröffentlichungen 90

7.2.2 Kongressbeiträge 91

7.3 Eidesstattliche Erklärung 94

7.4 Lebenslauf 95

7.5 Danksagung 96

1 Einleitung und Aufgabenstellung

Die Zielstellung dieser Arbeit war es, die Bindung des Thrombozytenproteins Plättchenfaktor 4 (PF4) an Zellen zu charakterisieren und darüber neue biologische Funktionen von PF4 zu bestimmen.

Als ein Mitglied der Familie der CXC-Chemokine wird PF4 auch als CXCL4 bezeichnet. PF4 ist ein 7.8 kDa großes Protein bestehend aus 70 Aminosäuren (**Abb. 1**). In Form von Tetrameren mit etwa 32 kDa wird er in den alpha-Granula von Thrombozyten gespeichert [1]. PF4 ist stark positiv geladen, bedingt durch einen äquatorial angeordeneten Ring aus positiv geladenen Aminosäureresten (Lysin, Arginin) [2]. Bei der Aktivierung von Thrombozyten kommt es zur Freisetzung von PF4 [3,4]. PF4 zirkuliert in nur sehr geringen Mengen frei im Blut, da er aufgrund der stark positiven Ladung schnell an negativ geladene Zelloberflächen über Glykosaminoglykane rekrutiert wird. So kann PF4 an die Thrombozytenoberfläche durch Interaktion mit Chondroitinsulfaten zurückbinden [5], über Heparansulfate an Endothelzellen gebunden werden [6,7] aber auch mit der Zellmembran von Neutrophilen [8,9] und Monozyten [10] interagieren. Auf der Endothelzelloberfläche wurde zusätzlich eine alternative Splicevariante des Chemokinrezeptors CXCR3, CXCR3-B, als funktioneller Rezeptor für PF4 charakterisiert [11]. Weiterhin ist beschrieben, dass PF4 auch in Monozyten [12] und T-Zellen [13] gebildet und sezerniert wird. Über die Funktion von PF4 ist bisher wenig bekannt. Gezeigt wurde bisher, dass PF4 einen hemmenden Einfluss auf die Hämatopoiese [14], Megakaryopoiese [15] und Angiogenese [16] hat, jedoch die Inflammation [17], Atherosklerose [18] und Thrombose [19] fördert.

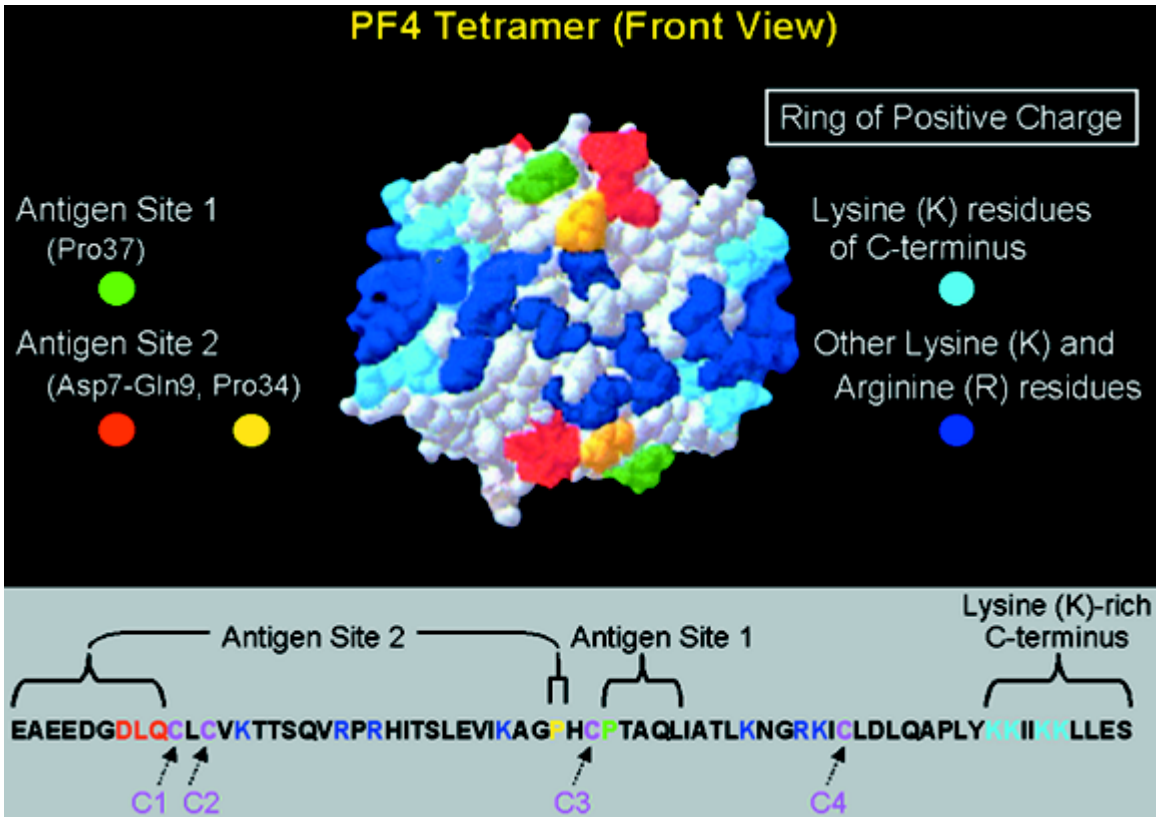

Abb. 1: Primäre (Monomer) und sekundäre (Tetramer) Struktur von PF4 (aus Warkentin et al., 2003 [20]).

Bekanntheit erlangte PF4 allerdings erst im Komplex mit dem Antikoagulans Heparin als das Hauptantigen der Heparin-induzierten Thrombozytopenie (HIT) [21]. Die Pathogenese der HIT stellt sich wie folgt dar (**Abb. 2**): Durch die ausgeprägte negative Ladung des Heparins können die positiv geladenen PF4-Tetramere stark angenähert werden, was die Ausbildung von Neoepitopen zur Folge hat [22]. Dadurch kommt es zur Anregung der Produktion von Antikörpern, welche die Komplexe binden und dadurch große Immunkomplexe generieren. Diese können die Thrombozyten über Kreuzvernetzung von FcγIIa-Rezeptoren aktivieren [23-25]. Es kommt zur Freisetzung von Thrombozyten-Mikropartikeln und anderen prokoagulatorischen Substanzen, wodurch Thrombingenerierung und Thrombusbildung ausgelöst werden [26,27]. Schließlich können diese Reaktionen zu Thrombozytopenie und lebensgefährlichen Thrombosen führen.

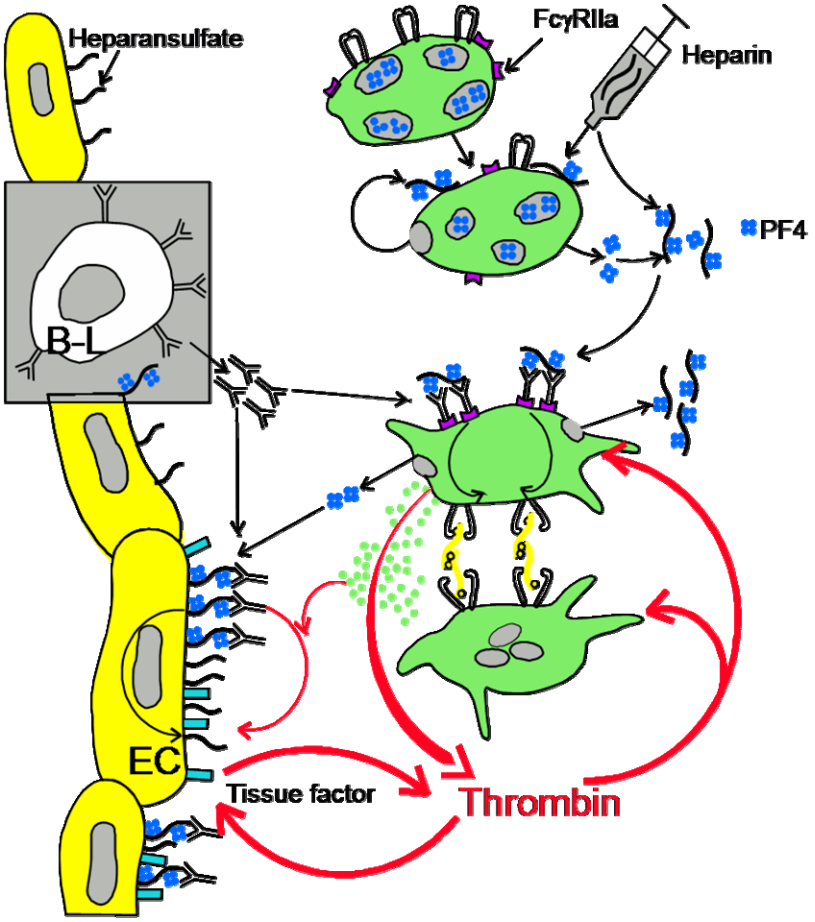

Abb. 2: Pathogenese der HIT (aus Greinacher und Lubenow, 2003 [28]).

Details zur HIT im medizinischen Kontext sind in der Übersichtsarbeit „A. Greinacher, K. Althaus, K. Krauel, and S. Selleng. Heparin-induced thrombocytopenia. Hämostaseologie. 2010; 30: 17-18, 20-28." (siehe 6.1) dargestellt.

Für die vorliegende Arbeit ist die empirische Beobachtung von Bedeutung, dass die Immunreaktion gegen PF4/Heparin-Komplexe eine Reihe von Besonderheiten aufweist.
Die Antikörperantwort der HIT folgt nicht dem klassischen Zeitverlauf wie etwa nach einer Immunisierung mit Vakzinen. Normalerweise wird nach erstem Antigenkontakt zunächst IgM gebildet, gefolgt von einer geringen Menge IgG (**Abb. 3**). Bei sekundärem Antigenkontakt hingegen kommt es zu einer schnelleren, stärkeren und länger anhaltenden IgG-Antwort mit einer zu vernachlässigenden IgM-Produktion Bei der HIT aber können anti-PF4/Heparin-Antikörper der Klasse IgG bereits 4-6 Tage nach erstmaliger Heparinexposition auftreten (**Abb. 4**). Weiterhin ist IgG die Antikörper-Hauptklasse, die bei der HIT gebildet wird ohne ein vorangehendes Auftreten von IgM [29,30]. Die Immunogenität von Heparin ist stark

abhängig vom pro-inflammatorischen Status des behandelten Patienten, wie etwa nach operativem Eingriff [31] oder schwerem Trauma [32]. Bis zu 50% der Patienten nach kadiochirurgischen und 20-30% der Patienten nach orthopädischen Operationen entwickeln anti-PF4/Heparin IgG in der zweiten Woche nach Heparinapplikation, wobei viele von ihnen zuvor nicht mit Heparin in Kontakt gekommen sind [33-36]. Eine seltene Form der HIT ist die „Delayed-onset HIT“, bei der Thrombozytopenien und Thrombosen erst einige Tage nach dem Beenden der Heparin-Therapie auftreten [37]. „Spontaneous HIT“ ist eine seltene Erkrankung, die der HIT klinisch und serologisch sehr ähnelt. Das Besondere an diesem Phänomen ist, dass die Patienten stark reaktive anti-PF4/Heparin IgG-Antikörper produzieren ohne zuvor mit Heparin behandelt worden zu sein [38-40]. Interessanterweise traten bei 3 der 5 beschriebenen Patienten kürzlich zurück liegende, bakterielle Infektionen auf. Dass anti-PF4/Heparin-Antikörper Merkmale von Autoantikörpern aufweisen wird zudem von der Beobachtung bei einigen Patienten unterstützt, dass sie auch ohne Anwesenheit von Heparin an Thrombozyten gebundenen PF4 erkennen können [41,42]. Eine weitere Besonderheit der Immunantwort der HIT ist, dass der Antikörperspiegel innerhalb weniger Wochen stark abfällt [29,43] und bei Reexposition mit Heparin kein Immungedächtnis wirksam wird [43]. Dazu passt, dass spezifische B-Gedächtnis-Zellen in Patienten, die nach kardiochirurgischem Eingriff anti-PF4/Heparin IgG entwickelten, nicht gefunden werden konnten [44].

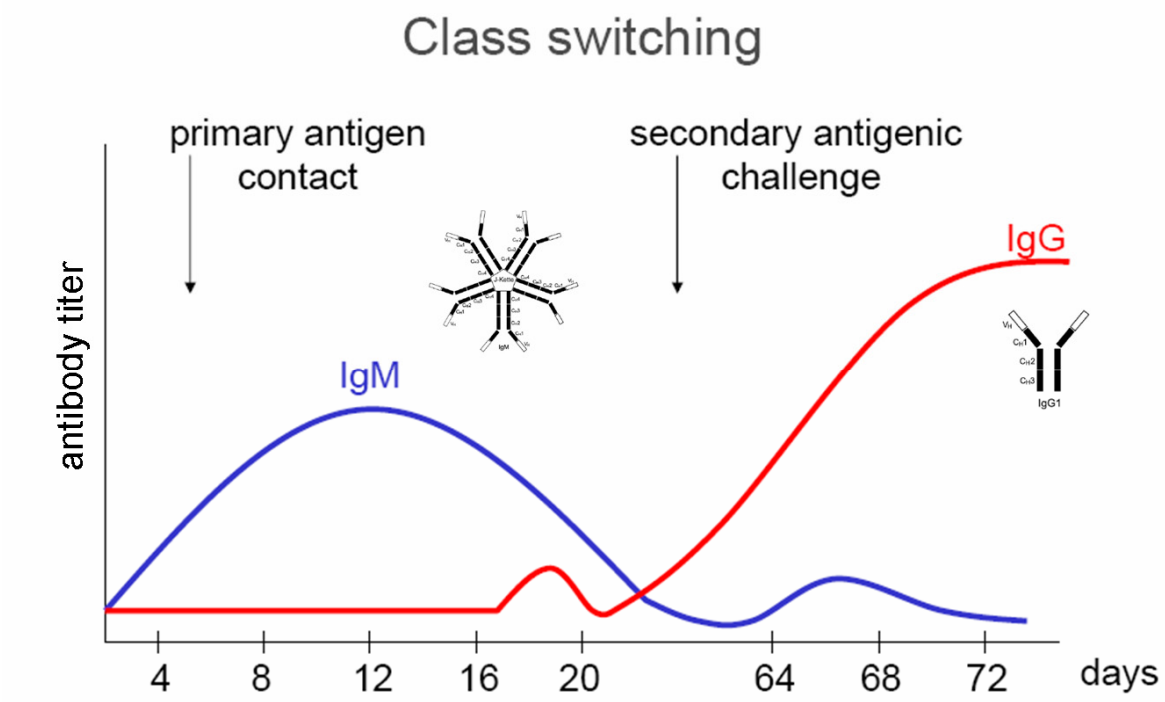

Abb. 3: Klassische Antikörperantwort nach primärem und sekundärem Antigenkontakt (verändert nach Schütt und Bröker, 2009 [45]).

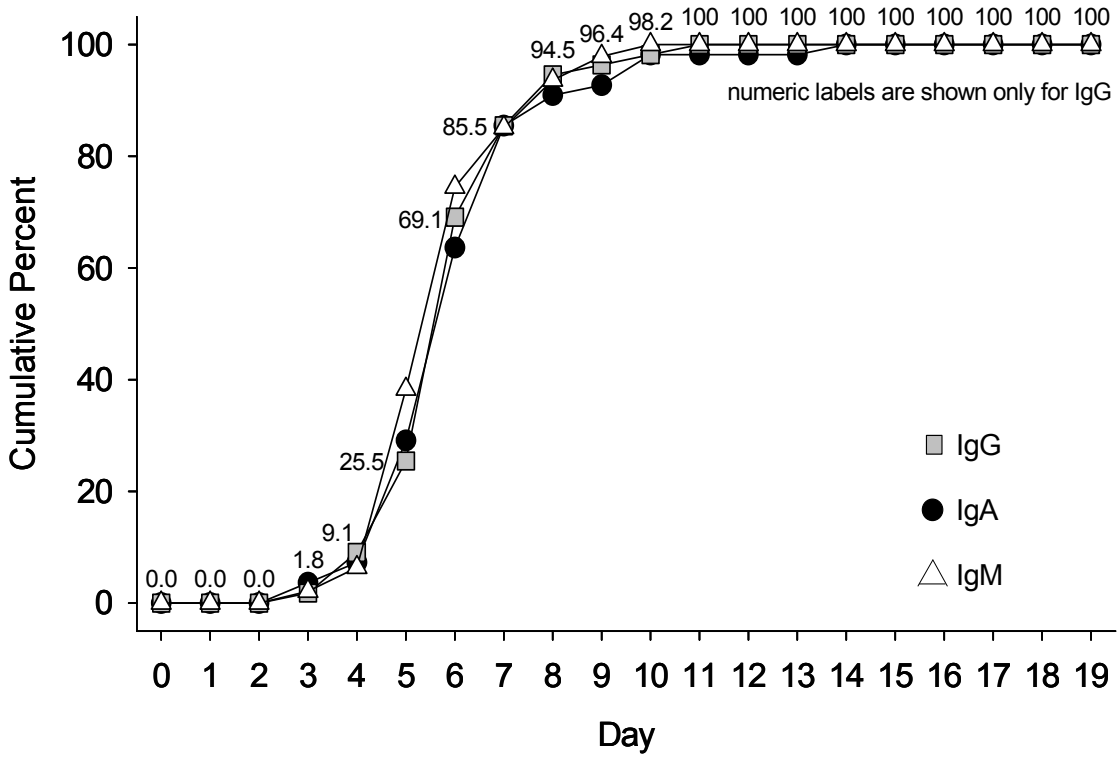

Abb. 4: Antikörperantwort der HIT nach erstmaliger Heparinexposition (aus Greinacher et al., 2009 [29]).

Thrombozyten sind nicht nur für die Blutgerinnung von großer Bedeutung. Aktuelle Studien weisen darauf hin, dass Thrombozyten auch bei der Abwehr von Pathogenen und der Modulation von Immunreaktionen wichtig sind (**Abb. 5**). In Wechselwirkung mit Endothelzellen und Leukozyten nehmen sie indirekt Einfluss auf die unspezifische und spezifische Abwehr. Zum einen sezernieren aktivierte Thrombozyten eine Reihe von Molekülen, die eine positive chemotaktische Wirkung auf Monozyten und Neutrophilen ausüben wie zum Beispiel PF4, ß-Thromboglobulin (ß-TG) und „Platelet-derived growth factor“ (PDGF) [46]. Zum anderen setzen Monozyten und Neutrophile Sauerstoffmetabolite, Platelet-Activating Factor (PAF) und antimikrobielle Substanzen wie Defensine frei, die die Thrombozyten stimulieren und deren Sekretion fördern. Besonders bei hohen Scherkräften sind adhärente Thrombozyten essentiell für die Rekrutierung von Leukozyten an Endothelzellen [47,48]. Sie können die Wanderung von Monozyten aus der Blutzirkulation in das entzündete Gewebe induzieren und modulieren [49]. Der direkte physische Kontakt resultiert vor allem aus der Interaktion von P-Selektin auf der Oberfläche aktivierter Thrombozyten mit auf den Immunzellen exprimiertem P-Selektin Glykoprotein Ligand-1 (PSGL-1) [50]. Die Adhäsion aktivierter Thrombozyten an Endothelzellen kann über die Brückenmoleküle Fibrinogen (Fbn) und von Willebrand-Faktor (vWF) erfolgen, welche die Integrine αIIbβ3 auf der Thrombozyten- und αvβ3 auf der Endothelzelloberfläche binden. Des Weiteren exprimieren aktivierte Thrombozyten den immunmodulierenden CD40-Liganden (CD40-L) [51]. Über die Bindung von CD40-L an CD40 auf Immunzellen induzieren Thrombozyten die Reifung von dendritischen Zellen sowie den Klassenwechsel und die antigenspezifische Antikörperproduktion von B-Zellen, was eine Modulation der adaptiven Immunität bewirkt [52]. Auch Endothelzellen und T-Lymphozyten werden über die CD40-CD40-L-Brücke stimuliert.

Thrombozyten sind nicht nur indirekt über Interaktion mit Gefäßwandzellen und professionellen Abwehrzellen an der Abwehr beteiligt. Thrombozyten selbst können antimikrobielle Substanzen freisetzen und auch direkt mit Pathogenen interagieren. Aktivierte Thrombozyten sezernieren eine Reihe hochpotenter antimikrobieller Peptide beziehungsweise Proteine wie zum Beispiel „regulated upon activation normal T-cell expressed and secreted“ (RANTES), Thymosin-β-4, „Platelet basic protein“ (PBP) und Fibrinopeptide A und B (FPA,B) [53,54]. Diese Abwehrsubstanzen greifen die Mikroorganismenmembran an, führen zur Porenbildung und depolarisieren somit die Membran [55]. Die direkte Interaktion von Thrombozyten mit Bakterien kann über die Bindung an bakterielle Oberflächenproteine

erfolgen oder über Plasmaproteine (zum Beispiel vWF, Fbn) hergestellt werden, die gleichzeitig an Rezeptoren auf der Thrombozytenoberfläche (zum Beispiel αIIbβ3) und Bakterienoberfläche (zum Beispiel „Staphyloccocal protein A“ [SpA], „Clumping factor A“ [ClfA]) binden [56]. Thrombozyten können auch über Expression von „Toll-like“-Rezeptoren an Pathogene binden [57,58]. Ebenfalls ist die Fähigkeit von Thrombozyten beschrieben, Pathogene durch Phagozytose zu beseitigen [59].

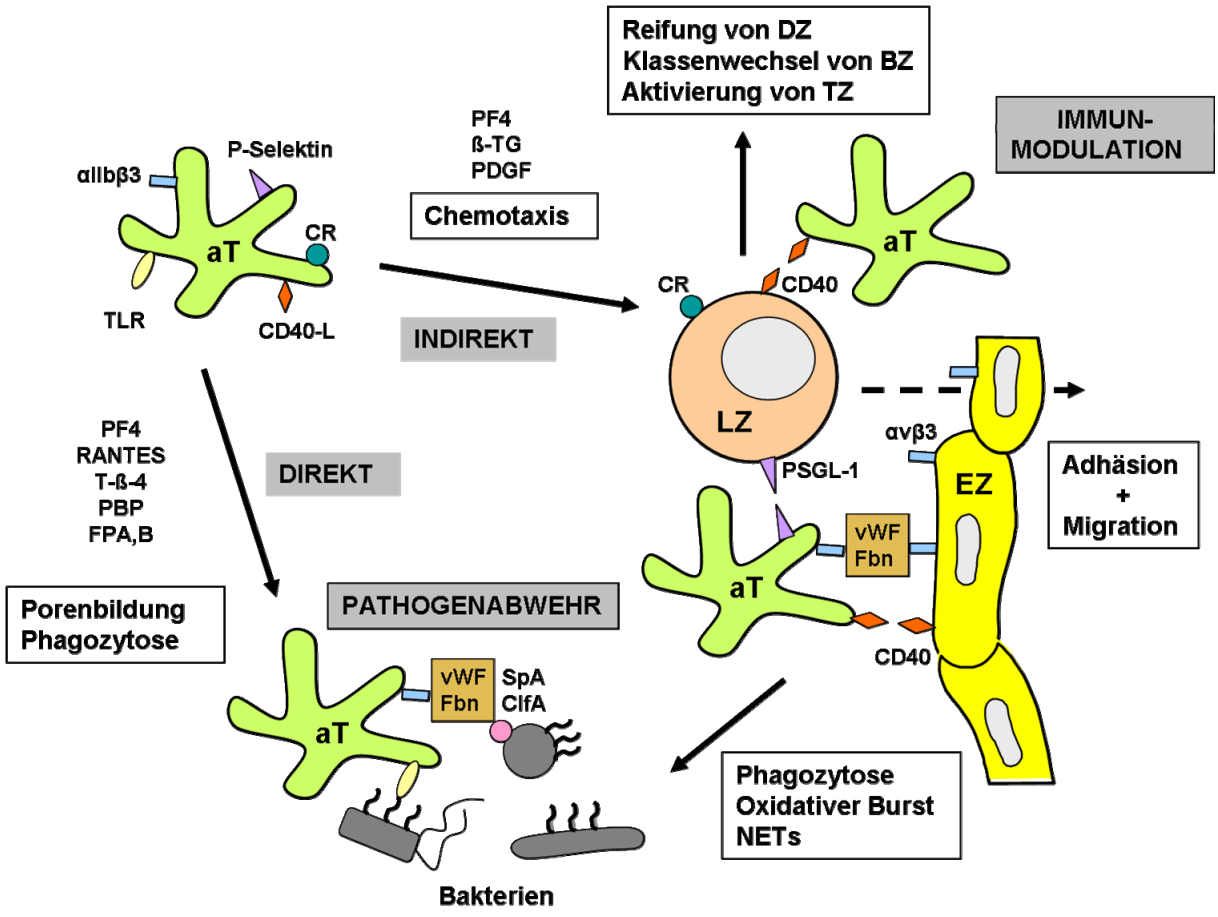

Abb 5.: Pathogenabwehr und Immunmodulation durch Thrombozyten. aT: aktivierter Thrombozyt; BZ: B-Zelle; CD40-L: CD40-Ligand; ClfA: „Clumping factor A“; CR: Chemokinrezeptor; DZ: Dendritische Zelle; EZ: Endothelzelle; Fbn: Fibrinogen; FPA,B: Fibrinopeptid A,B; LZ: Leukozyt; NETs: neutrophil extracellular traps; PBP: “Platelet basic protein”; PDGF: „Platelet-derived growth factor“; PF4: Plättchenfaktor 4; PSGL-1: P-Selektin Glykoprotein Ligand-1; RANTES: „regulated upon activation normal T-cell expressed and secreted“; SpA: „Staphylococcal protein A“; T-ß-4: Thymosin-ß-4; ß-TG: ß-Thromboglobulin; TLR: „Toll-like“-Rezeptor; TZ: T-Zelle; vWF: von Willebrand-Faktor

Negativ geladene Strukturen gibt es auch auf der Oberfläche von Bakterien (**Abb. 6 und 7**). So finden sich zum Beispiel Teichonsäuren auf Gram-positiven [60] und Lipopolysaccharide (LPS) auf Gram-negativen Bakterien [61]. Daher lag die Hypothese nahe, dass PF4 ladungsabhängig an Bakterien bindet und darüber eine Bedeutung in der Immunabwehr haben könnte.

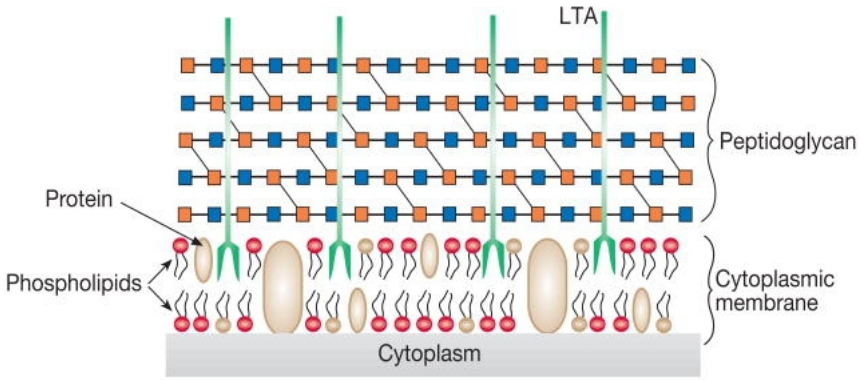

Abb. 6: Zelloberfläche Gram-positiver Bakterien. LTA: Lipoteichonsäure (aus Esko et al., 2009 [62]).

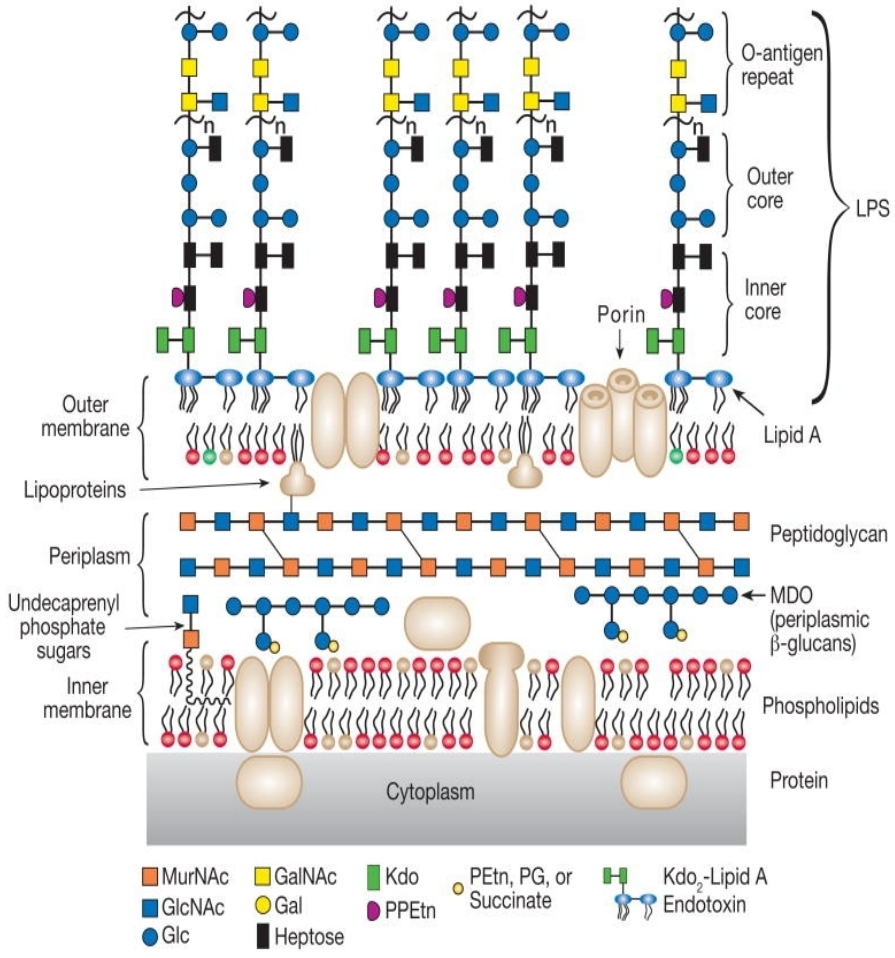

Abb. 7: Zelloberfläche Gram-negativer Bakterien. LPS: Lipopolysaccharid (aus Esko et al., 2009 [62]).

Im Einzelnen wurden in der vorliegenden Arbeit folgende Aufgabenstellungen bearbeitet:

1. Untersuchungen zur Interaktion von PF4 und Thrombozyten bzw. HEK-Zellen und deren Beeinflussung durch Polyanionen (**Krauel et al JTH 2008**)

2. Untersuchungen zur Interaktion von PF4 und Bakterien (**Krauel et al BLOOD 2011**)

3. Untersuchungen zur Bindung von humanen anti-PF4/Heparin-Antikörpern an PF4-beladene Bakterien und die Beeinflussung der Phagozytose von Bakterien *in vitro* (**Krauel et al BLOOD 2011**)

4. Etablierung eines Tiermodells zur Induktion von anti-PF4/Heparin-Antikörpern (**Krauel et al BLOOD 2011**)

5. Nachweis einer Assoziation zwischen chronischer bakterieller Infektion und Prävalenz von anti-PF4/Heparin-Antikörpern beim Menschen (**Greinacher et al BLOOD 2011**)

6. Zusammenfassung der Ergebnisse in einem Modell, das die ungewöhnlichen Charakteristika der Immunreaktion gegen PF4/Heparin-Komplexe als fehlgeleitete bakterielle Abwehrreaktion erklärt (**Krauel et al BLOOD 2011**)

2 Methoden, Ergebnisse und Diskussion

Die verwendeten Methoden sowie die Ergebnisse dieser Arbeit sind detailliert in den Publikationen (siehe Punkt 6) dargelegt. In dieser Zusammenfassung werden die wichtigsten Ergebnisse nochmals im Kontext der aktuellen Literatur dargestellt, vor allem aber auch die kritischen Punkte der Experimente sowie die Herausforderungen bei der Etablierung der Methoden erläutert. Diese Aspekte konnten in den Publikationen aufgrund der Längenvorgaben nicht ausgiebig adressiert werden.

Als erstes wurde die Interaktion von PF4 mit der Thrombozytenoberfläche in Abhängigkeit von Polyanionen mittels durchflusszytometrischer Analyse untersucht (**Abb. 8**). Da Voruntersuchungen ergeben haben, dass die PF4-Bindung an Thrombozyten durch Anwesenheit von Plasma gehemmt wird, wurden gelfiltrierte Thrombozyten aus antikoaguliertem Vollblut gesunder Probanden präpariert und mit humanem PF4 allein oder in Anwesenheit von drei antikoagulatorischen Polysacchariden, dem unfraktioniertem Heparin (UFH), dem Heparinoid Danaparoid (bestehend aus 84% Heparansulfat, 12% Dermatansulfat, 4% Chondroitinsulfat) oder dem Pentasaccharid Fondaparinux inkubiert. Nach Fixierung wurden die Thrombozyten gewaschen und mit einem polyklonalen Kaninchen-anti-human PF4-Antikörper inkubiert, der zuvor mittels eines FITC-Protein-Labeling-Kits fluoreszenzmarkiert wurde. Nach erneutem Waschen erfolgte die Messung der PF4-Bindung im Durchflusszytometer. In diesem Ansatz sollte geprüft werden, ob Polyanionen die PF4-Bindung an Thrombozyten verstärken können.

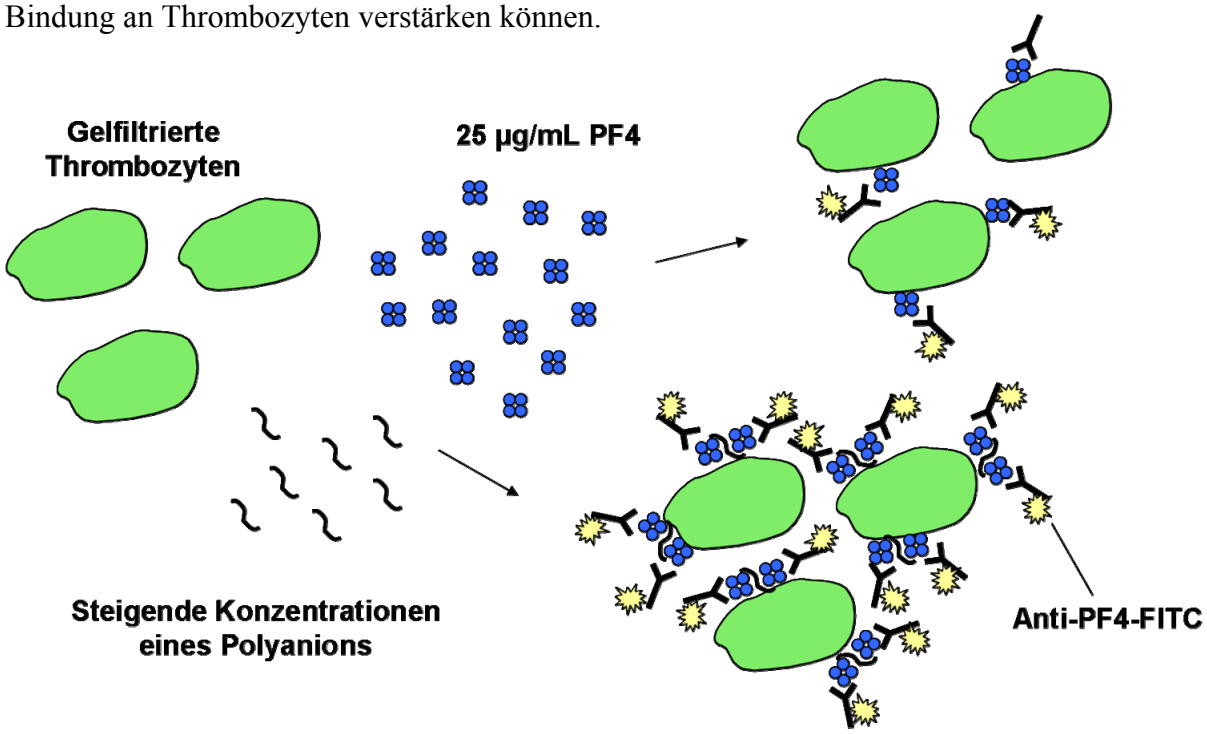

Abb. 8: Versuchsaufbau zur PF4-Bindung an Thrombozyten in Abhängigkeit von Polyanionen.

PF4 hat allein an die Thrombozyten gebunden. Diese Ausgangsbindung wurde als 100% definiert. Geringe Konzentrationen von UFH erhöhten die PF4-Bindung, wohingegen höhere Konzentrationen die PF4-Bindung erniedrigten (**Abb. 9**). Danaparoid steigerte die PF4-Bindung nur geringfügig und konnte bereits in geringeren Konzentrationen die PF4-Bindung hemmen. Fondaparinux hingegen konnte keine Erhöhung der PF4-Bindung bewirken und erniedrigte die PF4-Bindung nur in sehr hohen Konzentrationen.

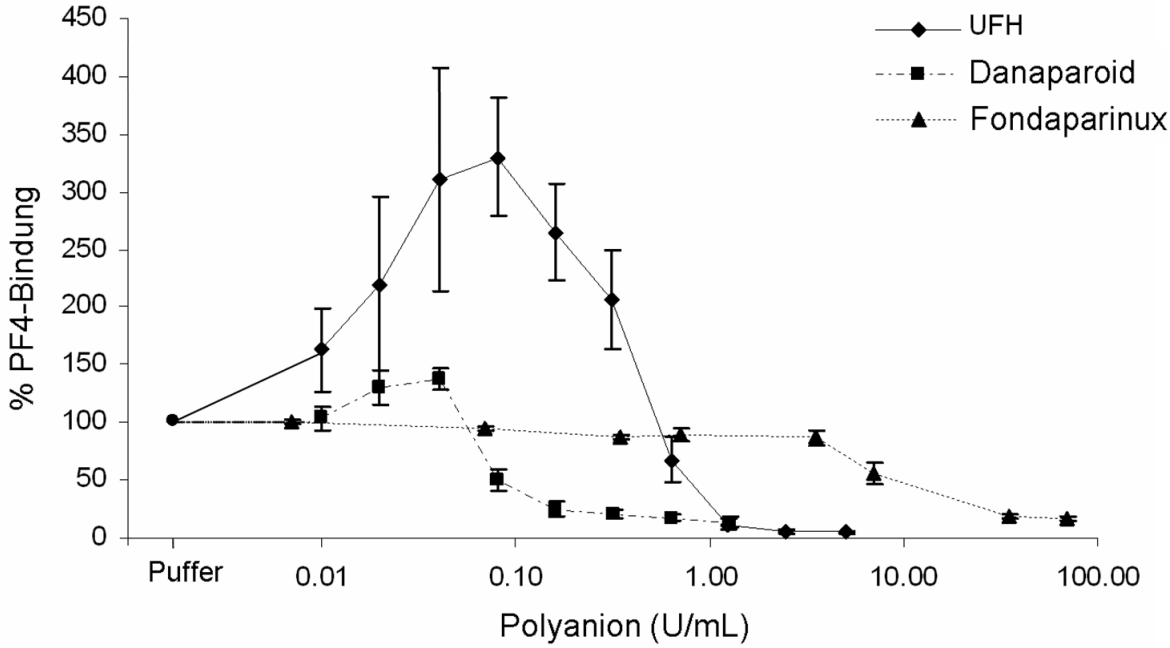

Abb. 9: PF4-Bindung an Thrombozyten in Abhängigkeit von Polyanionen.

Damit wurde gezeigt, dass die PF4-Bindung an Thrombozyten nicht von der antikoagulatorischen Aktivität dieser Medikamente (Fondaparinux >> UFH > Danaparoid) [63], sondern ladungsabhängig ist. Die Verstärkung der PF4-Bindung durch UFH und Danaparoid resultiert vermutlich aus der Fähigkeit dieser Antikoagulantien Komplexe mit PF4 zu bilden. Dadurch kommt es zur Aneinanderreihung vieler PF4-Tetramere was wahrscheinlich zu einer höheren Avidität der Komplexe zur Thrombozytenoberfläche führt. Da UFH aufgrund des höheren Sulfatierungsgrades eine stärkere Bindungsaffinität zu PF4 besitzt als die Bestandteile von Danaparoid (Heparin > Heparansulfat > Dermatansulfat > Chondroitinsulfat) [64] ist die Verstärkung durch Danaparoid vermutlich nicht so ausgeprägt. Fondaparinux ist zwar auch negativ geladen und kann mit PF4 interagieren und Cluster bilden [22], aber die Pentasaccharidsequenz reicht nicht aus um eine optimale Komplexformation mit PF4 generieren zu können [65].

Im nächsten Schritt wurde untersucht, wie Polyanionen die PF4/Heparin-Komplexbindung an Thrombozyten beeinflussen (**Abb. 10**). Für dieses Experiment wurden gelfiltrierte Thrombozyten zunächst mit PF4/Heparin-Komplexen beladen und dann gewaschen um nicht gebundenen PF4 und UFH zu entfernen. Anschließend erfolgte die Inkubation mit steigenden Polyanionkonzentrationen. Hierbei sollte getestet werden, ob die Polyanionen in der Lage sind PF4/Heparin-Komplexe von der Thrombozytenoberfläche abzulösen.

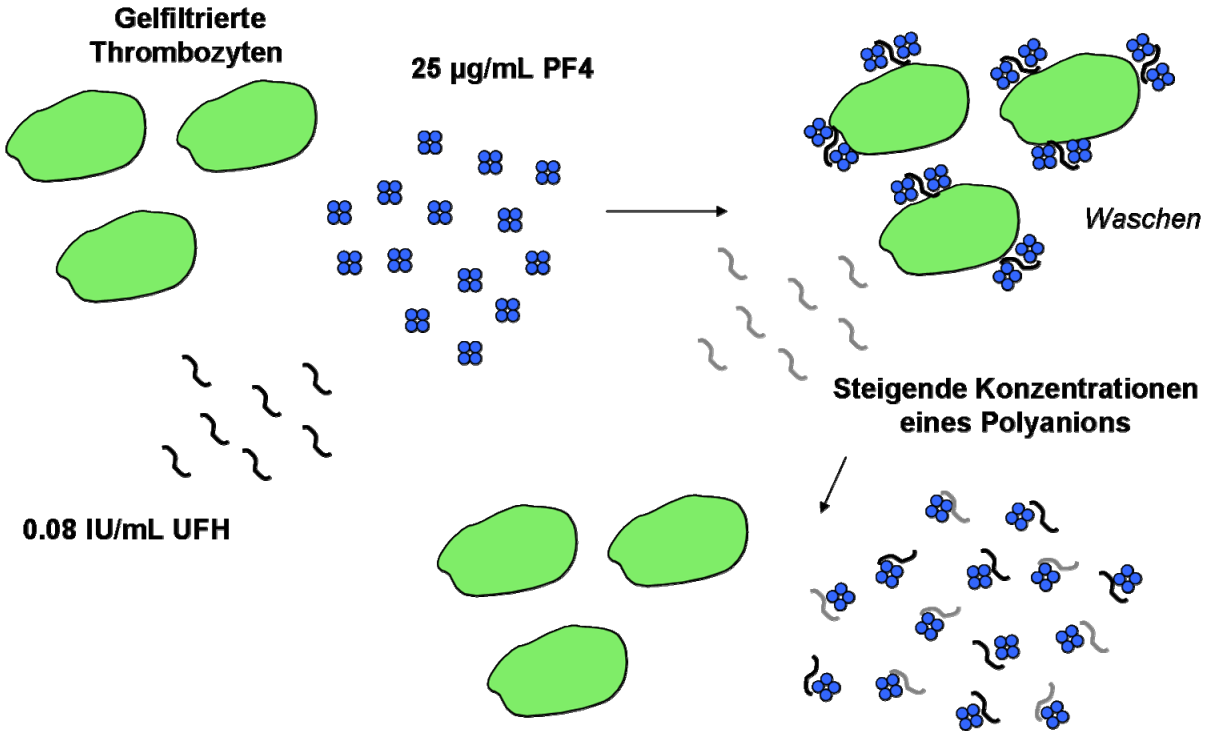

Abb. 10: Versuchsaufbau zum Einfluss von Polyanionen auf die PF4/Heparin-Komplexbindung an Thrombozyten

Die PF4/Heparin-Komplexbindung ohne Anwesenheit eines Polyanions wurde 100% gleichgesetzt. UFH und Danaparoid waren gleichermaßen in der Lage PF4/Heparin-Komplexe von der Thrombozytenoberfläche abzulösen, wohingegen Fondaparinux keinen Effekt zeigte (**Abb. 11**). Als Kontrolle wurden hier auch die direkten Thrombininhibitoren Hirudin, Bivalirudin und Argatroban mitgeführt, die aufgrund ihres strukturellen Aufbaus nicht mit PF4 interagieren sollten. Wie zu erwarten hatte keine dieser 3 Substanzen einen Einfluss auf die PF4-Bindung an Thrombozyten.

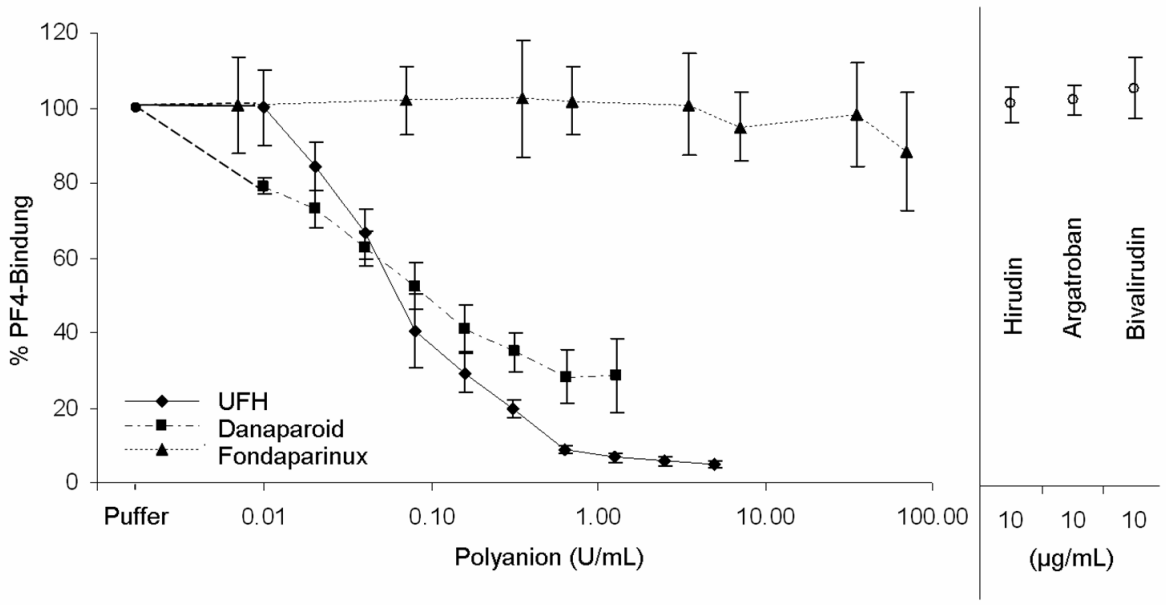

Abb. 11: PF4/Heparin-Bindung an Thrombozyten in Abhängigkeit von Polyanionen

Für die PF4/Heparin-Komplexbildung ist ein bestimmtes Verhältnis von PF4 zu Heparin notwendig [65]. Ein Überschuss von Heparin führt zur Auflösung der Komplexe. Daher ist nicht verwunderlich, dass steigende Konzentrationen von UFH die PF4/Heparin-Komplexbindung konzentrationsabhängig verringerte. In einem in diesem Teil der Arbeit nicht extra aufgeführten Versuch unter Anwendung der Photonenkorrelationsspektroskopie (PCS) konnte gezeigt werden, dass die Ablösung der PF4/Heparin-Komplexe durch Danaparoid von der Thrombozytenoberfläche auch über die Auflösung der Komplexe bewirkt wird (siehe 6.2, Krauel et al JTH 2008, Fig. 4).

Da PF4 nicht nur an Thrombozyten, sondern auch an anderen Zellen bindet wie zum Beispiel Endothelzellen [6,7] und Monozyten [10], wurde der Einfluss von Polyanionen auf die PF4-Bindung auch an einem alternativem Zelltyp getestet. Dafür wurde eine „Human embryonal kidney“(HEK)-Zelllinie verwendet, die mit PF4 transfiziert wurde und den PF4-Rezeptor CXCR3-B koexprimiert [13]. Nach Sekretion von PF4 kann dieser somit an die Zellmembran zurückbinden. Für das Experiment wurden HEK-Zellen mit steigenden Konzentrationen von Polyanionen inkubiert und, wie bereits für die Thrombozyten aufgeführt, hinsichtlich der PF4-Expression in der Durchflusszytometrie analysiert (**Abb. 12**). Die Hypothese war, dass auch in diesem System UFH und Danaparoid PF4 ablösen können, während Fondaparinux und die drei direkten Thrombininhibitoren keine Wirkung zeigen sollten.

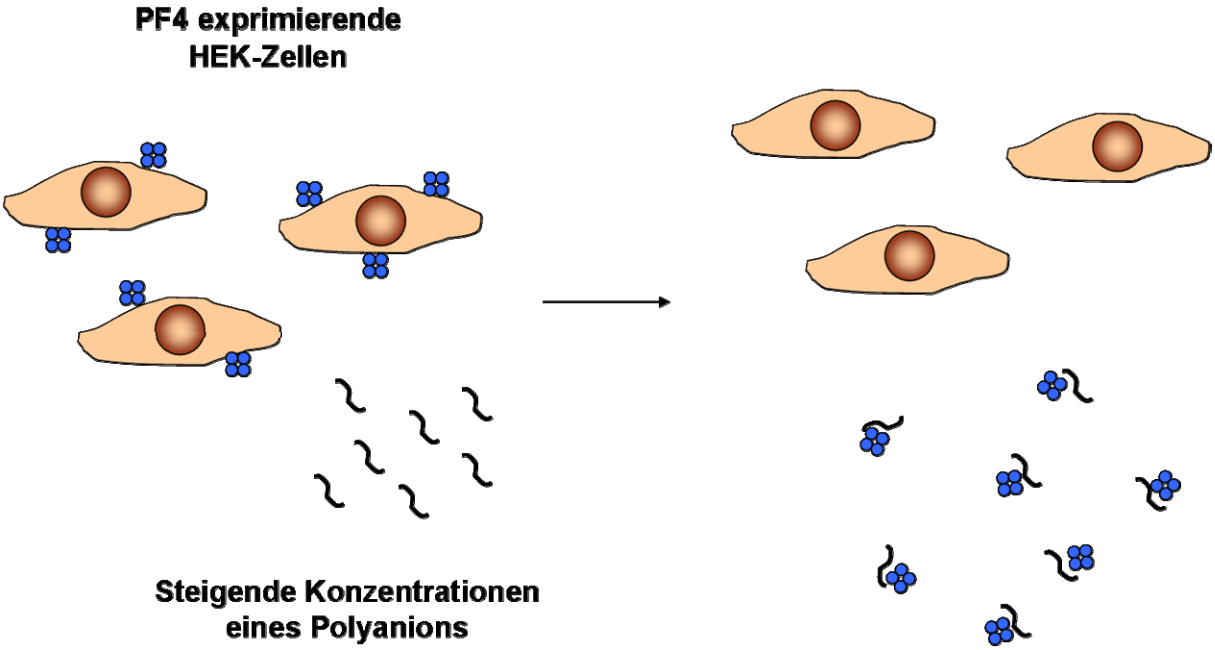

Abb. 12: Versuchsaufbau zum Einfluss von Polyanionen auf PF4 exprimierende HEK-Zellen.

Das Ausgangssignal von an den HEK-Zellen gebundenem PF4 ohne Zugabe eines Polyanions wurde wie zuvor 100% gleichgesetzt. Ähnlich zum Thrombozytenexperiment konnten UFH und Danaparoid PF4 von der Zelloberfläche ablösen, wohingegen Fondaparinux, Hirudin, Argatroban und Bivalirudin keinen Effekt hatten (**Abb. 13**).

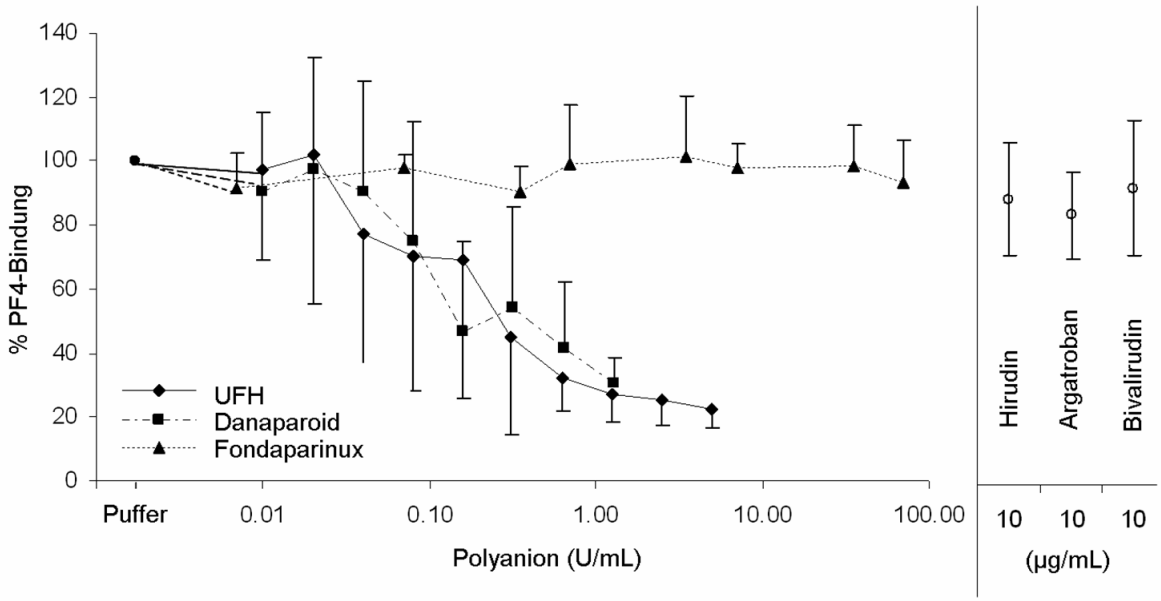

Abb. 13: Einfluss von Polyanionen auf PF4 exprimierende HEK-Zellen

Nachdem durch die Untersuchung der Interaktion von PF4 mit Thrombozyten und einem alternativem Zellsystem in Abhängigkeit von Polyanionen die Methode zur Untersuchung der Bindung von PF4 an Zellen erfolgreich etabliert war, wurde der Fokus auf die Interaktion von PF4 mit Bakterien gelegt. Dabei ergab sich das Problem, dass sich die zuvor für die Thrombozyten und HEK-Zellen eingesetzte Methode für die Bakterien nicht eignete. Der FITC-markierte anti-PF4-Antikörper wurde über Immunglobulin-bindende Proteine auf der Bakterienoberfläche, die mit der Fc-Region von Antikörpern wechselwirken [66], gebunden. Zum anderen enthält ein polyklonales Kaninchenantiserum Antikörper, die nicht nur gegen PF4 sondern auch generell gegen Strukturen auf der Bakterienoberfläche gerichtet sind. Die Lösung war, PF4 zu biotinylieren und mit fluoreszenzmarkiertem Streptavidin zu detektieren. Die Biotinylierung erfolgte nach einem Protokoll von Newman et al. [67]. Bei der Biotinylierung von PF4 war ein entscheidender Schritt, PF4 zunächst mit Heparin-Sepharose vorzuinkubieren, um die Heparinbindungsstellen am PF4-Molekül zu schützen (**Abb. 14**). Erst dann erfolgte die Inkubation mit Biotin und schließlich die Elution des biotinylierten PF4 von der Heparin-Sepharose durch einen Puffer mit hohem Salzgehalt. Damit war sichergestellt, dass die Bindungsstelle des PF4 für negativ geladene Polyanionen nicht durch Biotin alteriert wurde.

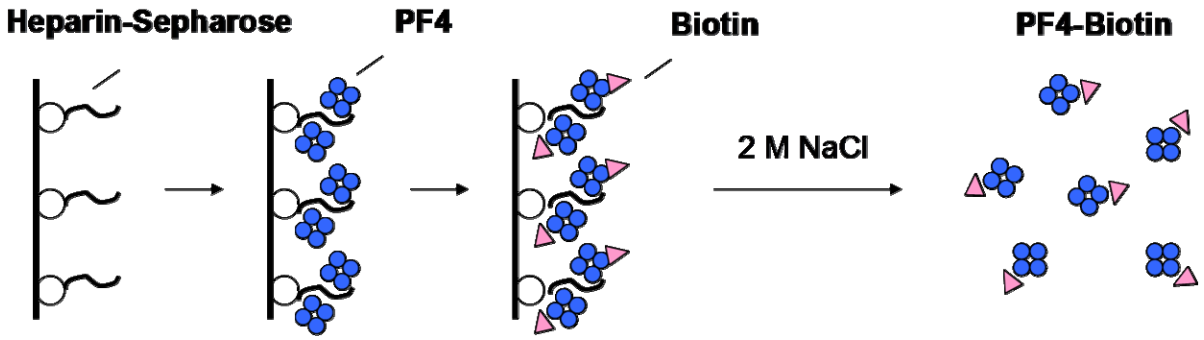

Abb. 14: Biotinylierung von PF4 nach Newman et al. [67].

Im eigentlichen Experiment wurden Bakterien bis zur mittleren logarithmischen Wachstumsphase kultiviert, mit steigenden Konzentrationen von biotinyliertem PF4 inkubiert, gewaschen, mit fluoreszenzmarkiertem Streptavidin inkubiert, wiederum gewaschen, fixiert und abschließend in der Durchflusszytometrie analysiert (**Abb. 15**).

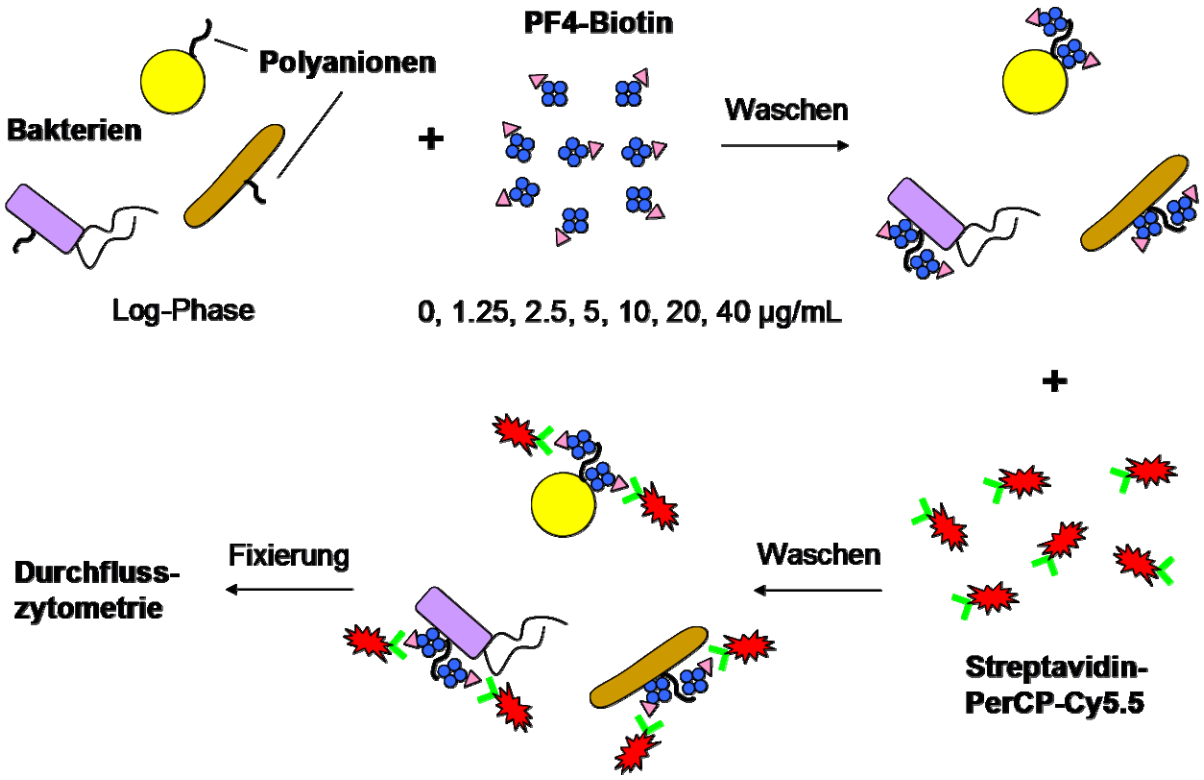

Abb. 15: Methode zur Messung der PF4-Bindung an Bakterien.

Im Ergebnis hat humaner PF4 (hu-PF4) konzentrationsabhängig an die Gram-positiven Bakterien *Staphylococcus aureus* (*S. aureus*), *Streptococcus pneumoniae* (*S. pneumoniae*) und *Listeria monocytogenes* (*L. monocytogenes*) als auch an die Gram-negativen Bakterien *Escherichia coli* (*E. coli*) und *Neisseria meningitidis* (*N. meningitidis*) gebunden (**Abb. 16**). Dass auch muriner PF4 (mu-PF4) an die Bakterienoberfläche rekrutiert wird, wurde beispielhaft an *E. coli* und *S. aureus* gezeigt.

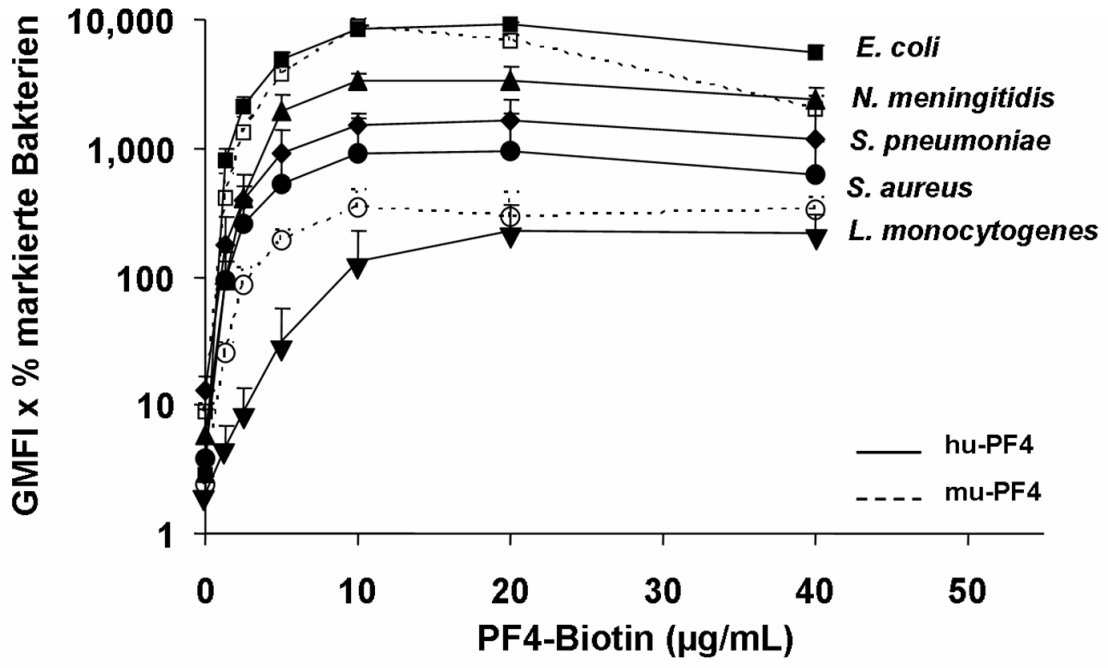

Abb. 16: PF4-Bindung an Bakterien.

In einem Folgeexperiment wurde auch hier der Einfluss von Polyanionen auf die PF4-Bindung am Beispiel von *S. aureus* getestet. Dafür wurde *S. aureus* bis zur mittleren logarithmischen Phase angezüchtet und mit PF4 in Gegenwart steigender Konzentrationen von UFH oder Dextransulfat inkubiert und wie bereits beschrieben hinsichtlich der PF4-Bindung analysiert (**Abb. 17**)**.** Sollte es sich hier ebenso um eine ladungsabhängige Bindung handeln, ist mit einer Inhibition der PF4-Bindung durch beide Substanzen zu rechnen.

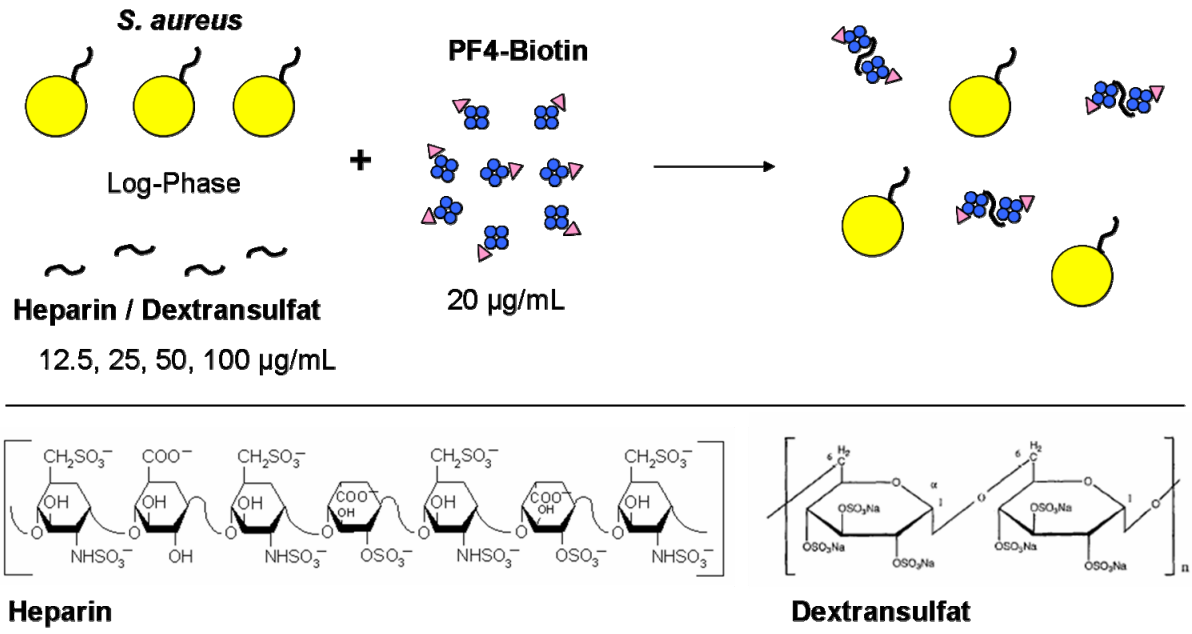

Abb. 17: Versuchsaufbau zum Einfluss von Polyanionen auf die PF4-Bindung an *S. aureus*.

Wie vermutet verringerte sich die PF4-Bindung an *S. aureus* mit zunehmender Konzentration von UFH oder Dextransulfat (**Abb. 18**). Damit war bewiesen, dass die Bindung von PF4 an Bakterien weitestgehend unabhängig von der Struktur des Polyanions ist.

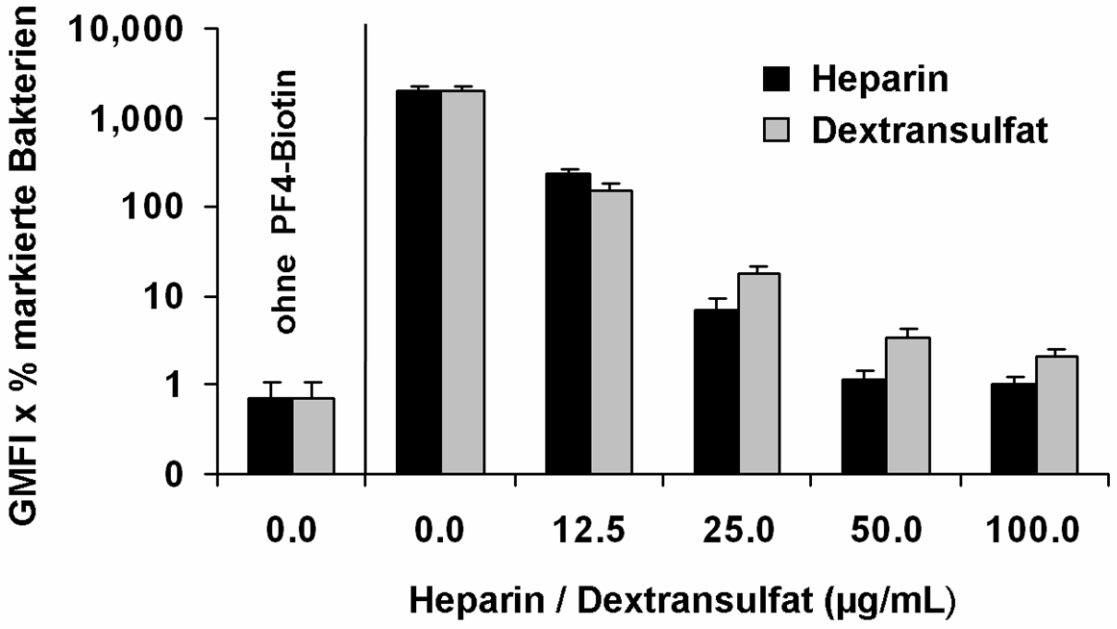

Abb. 18: PF4-Bindung an *S. aureus* in Abhängigkeit von Polyanionen.

Als nächstes sollte überprüft werden, ob durch die Bindung von PF4 an vermutlich negativ geladene Strukturen der Bakterienoberfläche PF4/Heparin-ähnliche Epitope generiert werden, die von anti-PF4/Heparin-Antikörpern erkannt werden können. Zur Bearbeitung dieser Fragestellung konnte die Bindung von Antikörpern aus humanen HIT-Seren an PF4-beladene Bakterien nicht einfach zum Beispiel über einen fluoreszenzmarkierten anti-human IgG-Antikörper mittels Durchflusszytometrie gemessen werden. Einerseits hätten die Antikörper über Fc-Rezeptoren auf der Bakterienoberfläche unspezifisch gebunden und andererseits enthalten humane Seren auch Antikörper, die gegen bakterielle Oberflächenmoleküle gerichtet sind. Gelöst wurde dieses methodische Problem über die Anwendung der Adsorptions-Elutions-Technik (**Abb. 19**). Bakterien (*S. aureus* oder *E. coli*) wurden entweder mit PF4 oder Puffer inkubiert und gewaschen, um nicht gebundenen PF4 zu entfernen. Dann wurden die vorbehandelten Bakterien mit anti-PF4/Heparin-Antikörper-haltigen Patientenseren inkubiert und wiederum gewaschen. Die anti-PF4/Heparin-Antikörper sollten idealerweise an die PF4-beladenen Bakterien binden und nach dem Waschschritt erhalten bleiben, während sie nicht an die nicht PF4-beladenen Bakterien binden sollten, im Überstand verbleiben und beim Waschen verloren gehen. Um gebundene Antikörper von der Bakterienoberfläche abzulösen erfolgte die Zugabe von saurem Glycin-Puffer,

gefolgt von einer Neutralisation mit basischem TRIS-Puffer. Schließlich wurden die Eluate im PF4/Heparin-ELISA [68] und Heparin-induziertem Plättchenaktivierungstest (HIPA) [69] analysiert. Beide Methoden dienen dem Nachweis von anti-PF4/Heparin-Antikörpern im Serum. Während der PF4/Heparin-ELISA nur die Bindung der Antikörper an PF4/Heparin-Komplexe beschichtet auf einer Mikrotiterplatte prüft, kann der Funktionstest HIPA darüber Aufschluss geben, ob die Antikörper auch in der Lage sind Thrombozyten zu aktivieren.

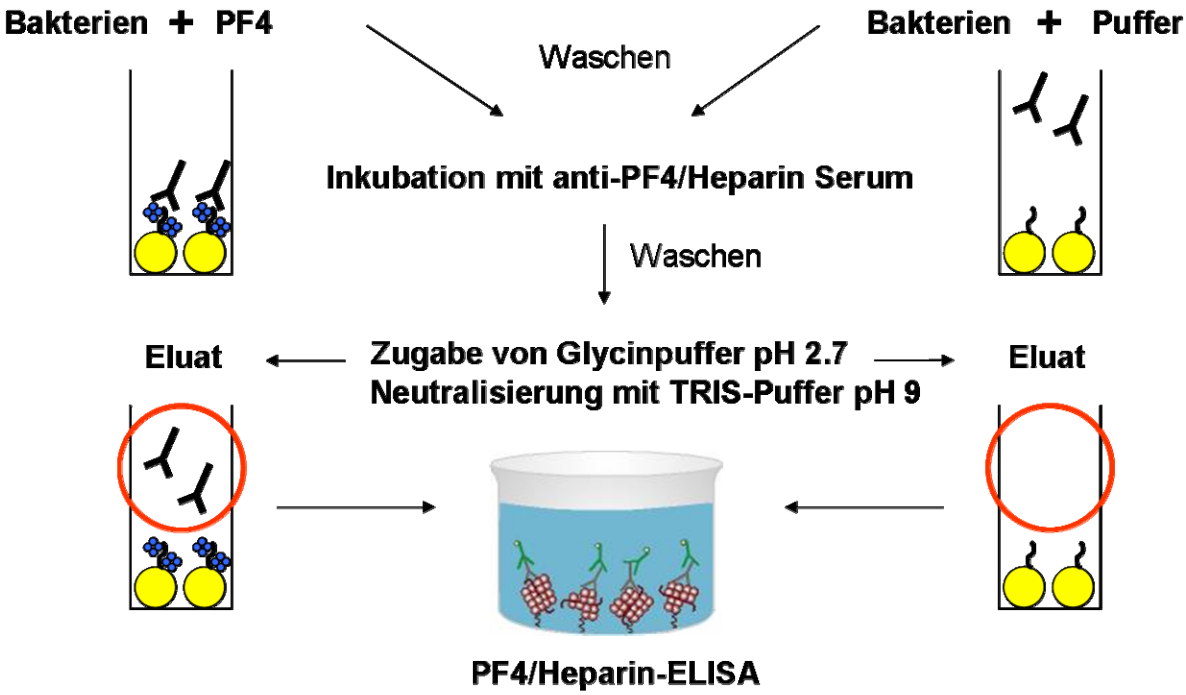

Abb. 19: Methode zur Analyse der Bindung von anti-PF4/Heparin-Antikörpern an PF4-beladene Bakterien.

Die Eluate von mit PF4 vorinkubierten Bakterien haben im PF4/Heparin-ELISA an PF4/Heparin-Komplexe gebunden, während die Eluate von nur mit Puffer vorbehandelten Bakterien negativ waren (**Abb. 20**, am Beispiel von *S. aureus*). Die Bindungsspezifität zeigte sich durch die Hemmbarkeit mit hoher Heparinkonzentration [70] sowie durch die ausbleibende Bindung an PF4 allein. Übereinstimmend zeigte sich, dass die Antikörper nach mehreren Adsorptionsschritten aus dem Serum entfernt werden konnten (Einfügung in **Abb. 20**). Zudem waren alle 5 Eluate von PF4-beladenen *E. coli*, die mit verschiedenen HIT-Seren gewonnen wurden, und 3 von 5 Eluaten von PF4-beladenen *S. aureus* im HIPA positiv.

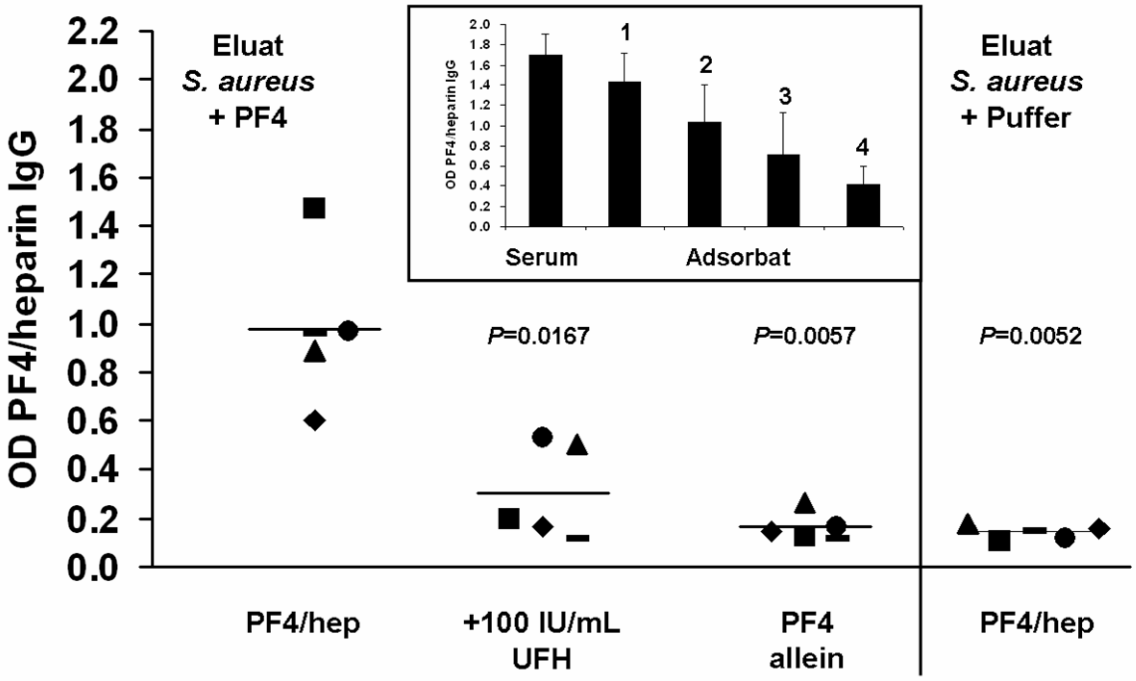

Abb. 20: Signal der Eluate von *S. aureus* im PF4/Heparin-ELISA.

An Pathogene gebundene Antikörper können Fc-Rezeptor-vermittelt eine Phagozytose durch „Fresszellen“ wie zum Beispiel neutrophile Granulozyten, die auch als polymorph nukleäre Zellen (PMN) bezeichnet werden, vermitteln und somit zur Beseitigung des Pathogens beitragen [71]. Ob an Bakterien gebundene anti-PF4/Heparin-Antikörper auch solch einen Abwehrmechanismus vermitteln können wurde im Folgenden getestet. Um den Effekt der anti-PF4/Heparin-Antikörper von dem anderer gegen die Bakterien gerichteter Antikörper im Patientenserum unterscheiden zu können wurden die Seren zuvor an nicht PF4-beladene Bakterien voradsorbiert, um die für diese Fragestellung nicht relevanten Antikörper zu entfernen. Da auch das Komplementsystem die Phagozytose beeinflussen kann [71], wurden die Seren neben der Voradsorption auch hitzeinaktiviert, was eine Zerstörung der Komplementfaktoren bewirkt [72]. FITC-markierte *E. coli* wurden mit PF4 oder Puffer vorinkubiert, gewaschen, dann mit dem vorbehandelten anti-PF4/Heparin-Antikörper-positiven Patientenserum, negativem Kontrollserum oder Puffer inkubiert und in einem Phagozytoseassay nach Lehmann et al. [73] analysiert (**Abb. 21**).

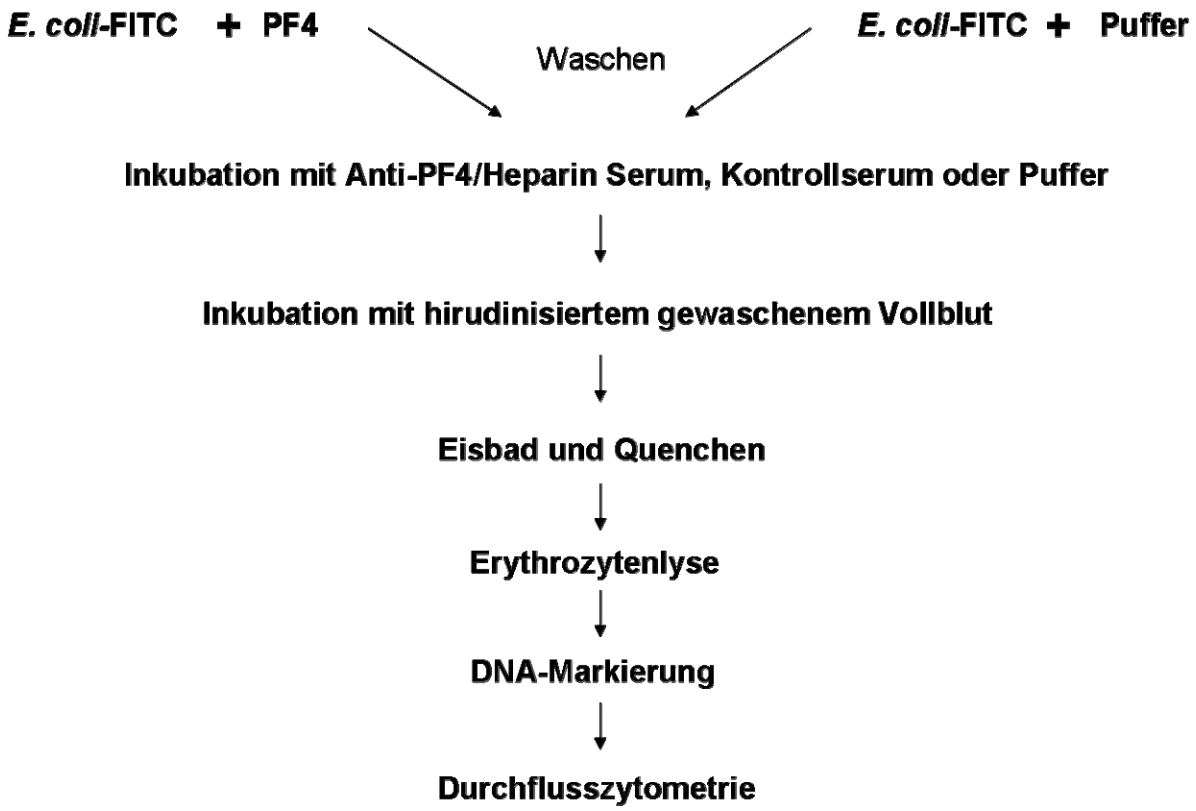

Abb. 21: Phagozytoseassay.

Tatsächlich konnte die Phagozytose von PF4-beladenen *E. coli* durch Vorinkubation mit anti-PF4/Heparin-Antikörper-haltigem Serum im Vergleich zu Kontrollserum und Puffer gesteigert werden (**Abb. 22**). Dieser Effekt war abhängig von der PF4-Vorbehandlung von *E. coli*.

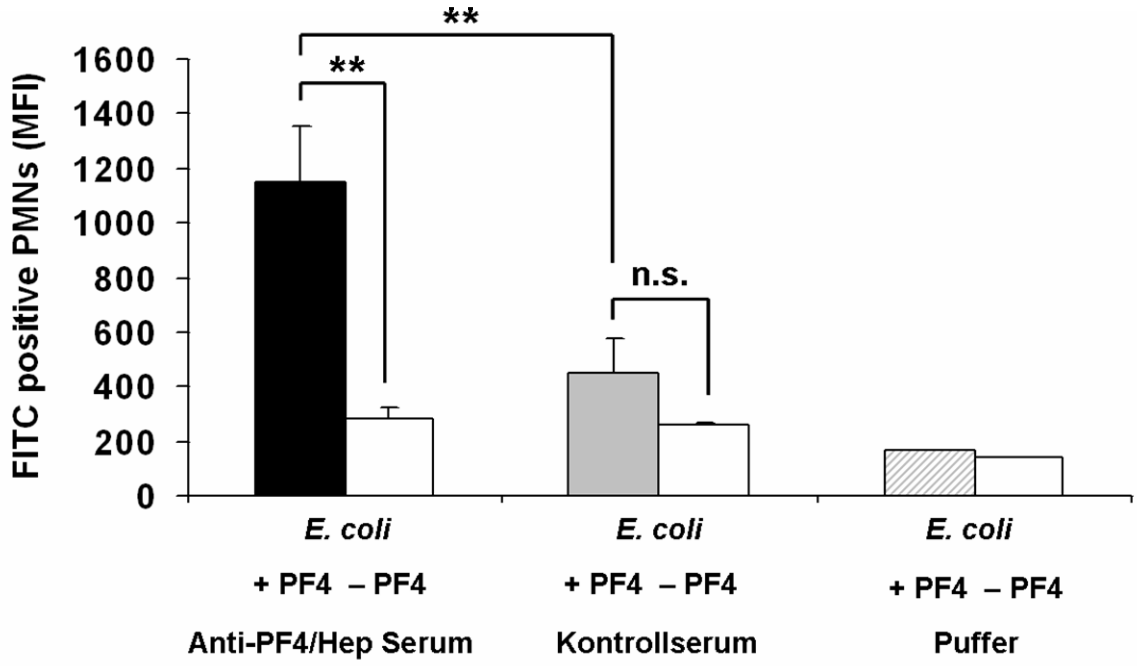

Abb. 22: Effekt von anti-PF4/Heparin-Antikörpern auf die Phagozytose von PF4-beladenen *E. coli*.

Die bisherigen Ergebnisse führten zu der Fragestellung, ob die Bildung von Antikörpern gegen komplexiertes PF4, welches zum Beispiel durch Bindung an die Bakterienoberfläche generiert wird, einen prinzipiellen Wirtsabwehrmechanismus darstellt und auch *in vivo* Bedeutung hat. Um dieses aufzuklären wurde das Maus-Sepsis-Modell Colon Ascendens Stent Peritonitis (CASP) [74,75] verwendet (**Abb. 23**). Bei diesem Tiermodell wird ein Stent in den aufsteigenden Colon implantiert um einen kontrollierten Übertritt von Faeces in die Abdominalhöhle zu gewährleisten. Daraus resultiert eine Peritonitis, die eine polymikrobielle Sepsis darstellt. Um von den Effekten unterscheiden zu können, die nur durch den chirurgischen Eingriff zustande kommen könnten, wurden auch Sham-Operationen durchgeführt. Dabei wird der Stent außerhalb des aufsteigenden Colons fixiert ohne einen Übertritt des Darminhaltes zu ermöglichen. Ein Tag bzw. 3, 7, 14 oder 28 Tage nach CASP- oder Sham-Operation wurden Mausseren gewonnen und im modifizierten PF4/Heparin-ELISA (rekombinanter [r-] muriner statt humaner PF4, Konjugate gegen murine statt humane Antikörper) hinsichtlich IgM und IgG getestet.

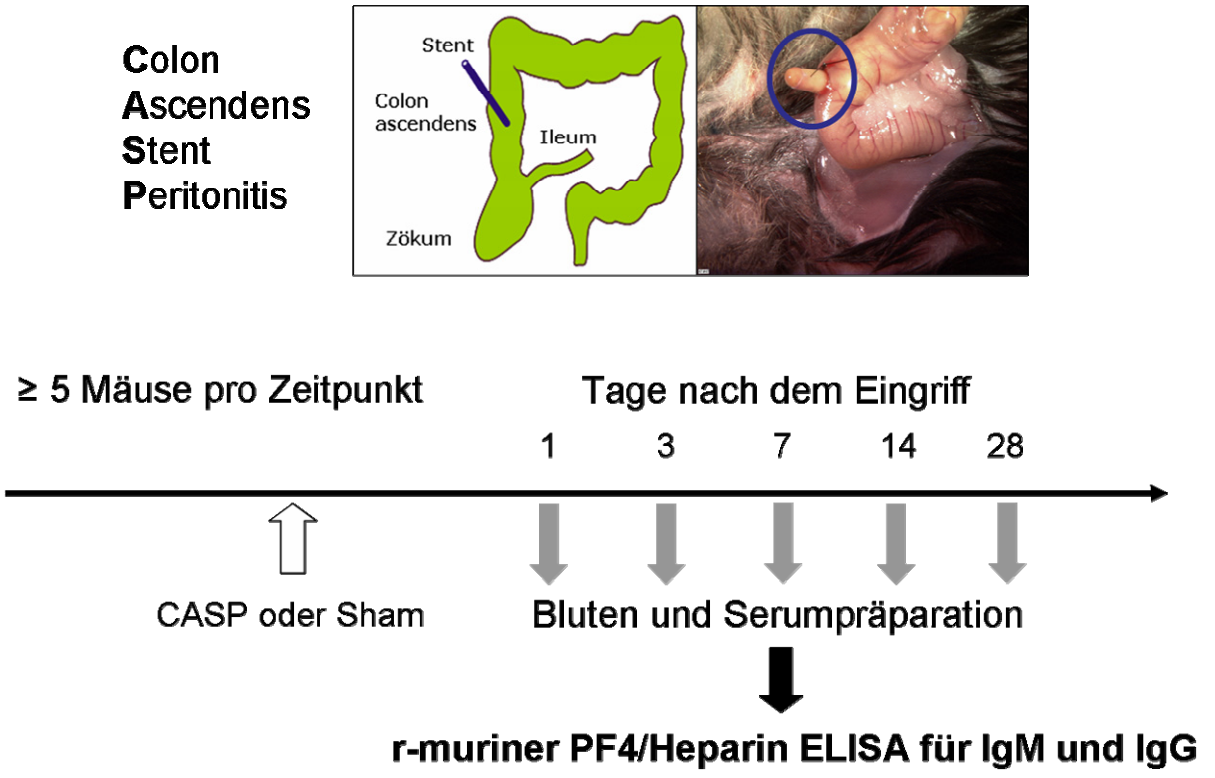

Abb. 23: Versuchsaufbau zur Analyse der anti-PF4/Heparin-Antikörperproduktion im CASP-Modell.

Mäuse entwickelten anti-PF4/Heparin-Antikörper der Klasse IgM beginnend vom 3. Tag und IgG beginnend vom 14. Tag nach der CASP-Operation (**Abb. 24**). Dieser Antikörperverlauf passt zur Immunreaktion nach erstmaligem Antigenkontakt, die sogenannte Primärantwort

(siehe **Abb. 3** in der Einleitung). Nicht in der Abbildung zu sehen ist, dass die Antikörperbindung in Gegenwart hoher Heparinkonzentration und bei Beschichtung mit PF4 allein signifikant reduziert war. Die Sham-operierten Mäuse zeigten eine nur sehr schwache, signifikant geringere Antikörperproduktion als die CASP-operierten Mäuse. Seren unbehandelter Mäuse zeigten kaum Signale.

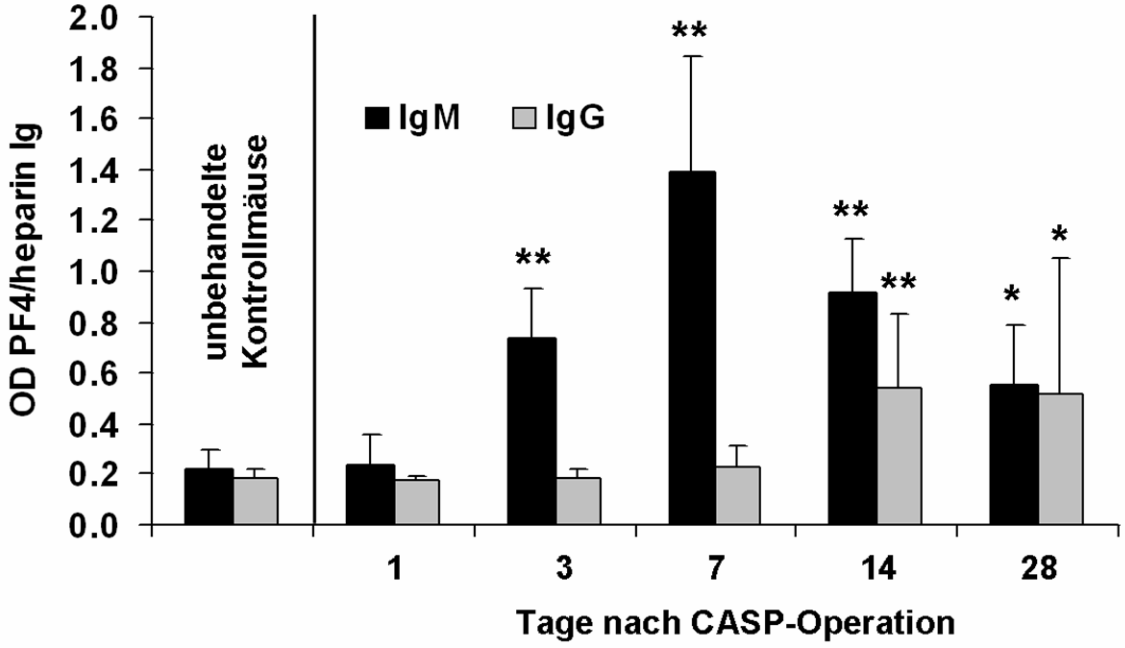

Abb. 24: Verlauf der anti-PF4/Heparin-Antikörperproduktion von Mäusen nach CASP-Operation.

Mit diesem Experiment konnte die Hypothese bekräftigt werden, dass die schnelle anti-PF4/Heparin-IgG-Antwort bei der HIT nach erstmaliger Heparinexposition wahrscheinlich eine sekundäre Immunantwort darstellt und die Primärantwort bereits durch vorangegangene bakterielle Infektionen ausgelöst wurde. Zusätzlich steht nun mit der CASP ein neues *in vivo* Maus-Modell für die HIT zur Verfügung, um die untypische Immunantwort gegen PF4/Polyanion-Komplexe untersuchen zu können. Es gibt zwar schon Maus-Modelle für die HIT, aber diese beruhen entweder auf einer „künstlichen" aktiven Immunisierung mit PF4/Heparin-Komplexen [76] beziehungsweise mit humanen anti-PF4/Heparin-Antikörpern zur Induktion von anti-anti-Idiotyp-Antikörpern [77] oder einer passiven Immunisierung durch Injektion eines monoklonalen Maus-Antikörpers namens KKO gerichtet gegen PF4/Heparin-Komplexe [25]. Eine Schwäche des CASP-Modells ist allerdings, dass bisher nur die primäre Immunantwort auf eine bakterielle Infektion gezeigt werden konnte. Mäuse wurden bislang nach CASP-Operation nicht mit Heparin behandelt, um zu sehen, ob sich dann eine schnelle

sekundäre Antwort entwickelt. In diesem Maus-Modell kann nur die Antikörperinduktion untersucht werden, nicht das Auftreten der beim Menschen typischen klinischen Manifestation mit Thrombozytopenie und Thrombosen, da Mäuse keinen zum Menschen analogen FcγIIa-Rezeptor auf Thrombozyten exprimieren [78], der für die Aktivierung von Thrombozyten durch Immunkomplexe in der HIT von entscheidender Bedeutung ist [23-25].

Da bakterielle Infektionen auch hin und wieder beim Menschen vorkommen oder gar chronisch sind lag die Vermutung nahe, dass auch in der Normalbevölkerung anti-PF4/Heparin-Antikörper zu finden sein müssten. Um diese Fragestellung zu beantworten wurden Seren von Normalprobanden, die bei der bevölkerungsbezogenen, epidemiologischen SHIP (study of health in pomerania)-Studie [79,80] in der Region Vorpommern gewonnen wurden, untersucht. Die Analyse von etwa 4000 Seren im PF4/Heparin-ELISA ergab, dass 18.8% der Normalbevölkerung anti-PF4/Heparin-Antikörper der Klasse IgM (**Abb. 25 A**) und 6.1% der Klasse IgG (**Abb. 25 B**) aufweisen (OD>0.5), wobei aufgrund der detaillierten erhobenen Anamnese in der SHIP Studie die Wahrscheinlichkeit bei vielen Probanden sehr gering war, dass sie zuvor in Kontakt mit Heparin gekommen waren.

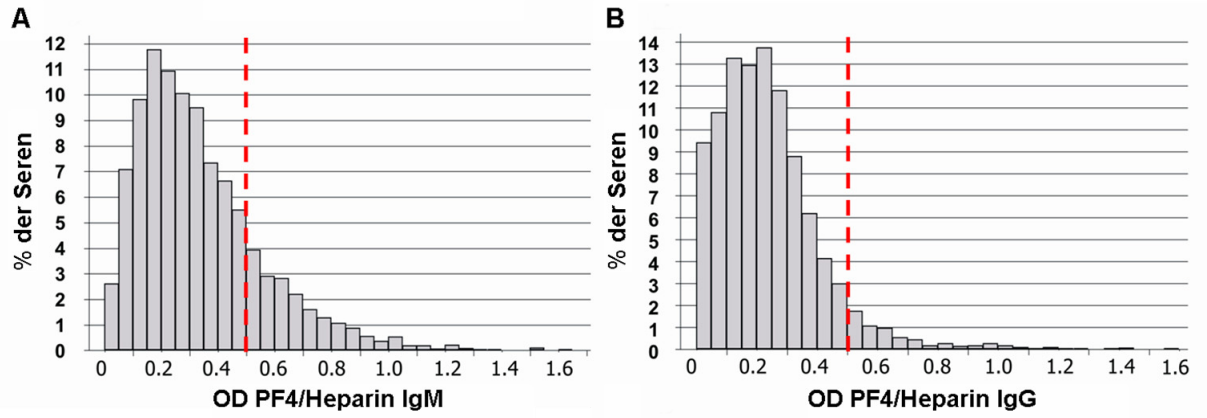

Abb. 25: Prävalenz von anti-PF4/Heparin-Antikörpern der Klasse IgM und IgG in der Normalbevölkerung.

Die Ursache für das Vorkommen von anti-PF4/Heparin-Antikörpern bei einem so hohen Prozentsatz von Probanden aus der Normalbevölkerung, sowie das schnelle Auftreten von IgG Antikörpern bei 50% der Patienten nach kardiochirurgischem Eingriff [36,81] kann nicht vorrangig eine Sepsis sein. Dafür ist die Sepsis, eine lebensbedrohliche Erkrankung, viel zu selten. Daher wurde analysiert, ob häufiger auftretende, chronische, bakterielle Infektionen eine Möglichkeit der Vorimmunisierung bieten. Dieses haben wir am Beispiel der Parodontitis, einer polymikrobiellen chronischen Infektion, untersucht. Die Parodontitis entspricht hinsichtlich der Prävalenz (15-20% der adulten Population mit schwerem

Kranheitsverlauf [82]) etwa dem Vorkommen der anti-PF4/Heparin-Antikörper in der Normalbevölkerung. Auch für diese Analyse konnten Daten der SHIP-Studie und einer weiteren Fall-Kontroll-Studie genutzt werden. Es hat sich tatsächlich gezeigt, dass das Auftreten von anti-PF4/Heparin-Antikörpern mit dem Parodontitis-Status assoziiert war. Um dies weiter zu untermauern, wurden mit den oben ausgeführten Methoden die PF4-Bindung an die parodontalpathogenen Bakterien *Aggregatibacter actinomycetemcomitans* (*A. a.*) bzw. *Porphyromonas gingivalis* (*P. g.*) untersucht (**Abb. 26 A**) und gezeigt, dass diese Anaerobier ebenso PF4 binden können, wie die in den früheren Experimenten untersuchten Modell-bakterien. In analogen Experimenten mit der Adsorptions-Elutions-Technik wurde dann auch gezeigt, dass anti-PF4/Heparin-Antikörper ebenfalls an diese PF4-beladenen Bakterien binden (**Abb. 26 B**). Damit ist bewiesen, dass diese Bakterien nach der PF4-Bindung das gleiche Epitop aufweisen, das entsteht, wenn PF4 und Heparin Komplexe bilden.

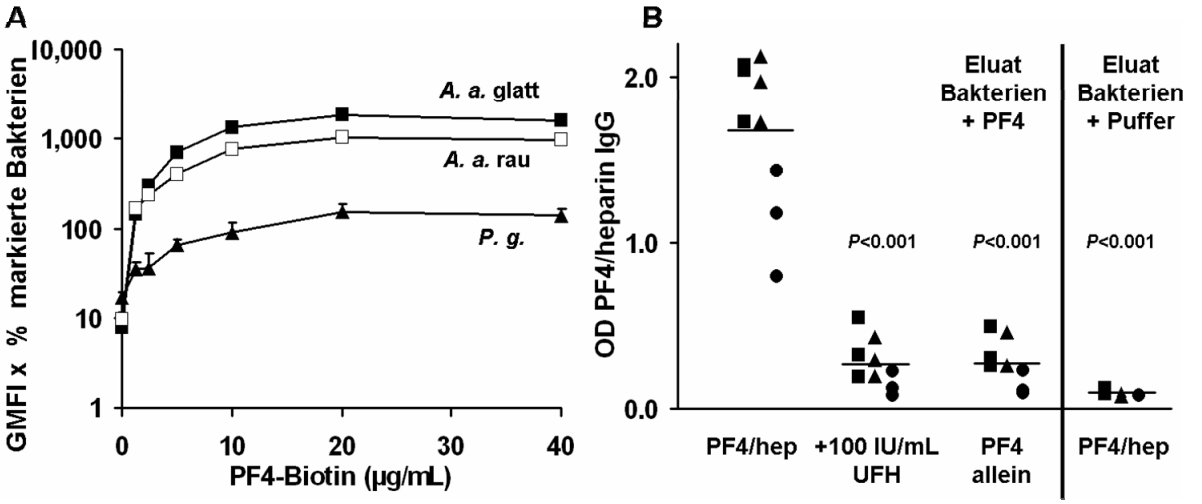

Abb. 26: PF4-Bindung an parodontalpathogene Bakterien sowie die dadurch ermöglichte Erkennung durch anti-PF4/Heparin-Antikörper aus Seren von HIT-Patienten.

Der folgende Abschnitt fasst die wichtigsten Ergebnisse dieser Arbeit zusammen (**Abb. 27**). Aktivierte Thrombozyten setzen positiv geladenen PF4 frei. Durch die Interaktion von PF4 mit negativ geladenen Strukturen auf der Bakterienoberfläche kommt es zur Ausbildung von Neoepitopen, welche die Produktion von PF4/Polyanion-spezifischen Antikörpern durch B-Zellen initiieren. Die Bindung dieser Antikörper an PF4-beladene Bakterien führt zur Opsonierung und erleichtert die Phagozytose der Bakterien durch zum Beispiel Granulozyten. Da gezeigt wurde, dass PF4 an eine Vielzahl unterschiedlicher Bakterien nur aufgrund von Ladungen bindet und nur eine Antikörperspezifität notwendig ist um PF4 gebunden an Bakterien zu erkennen, stellt dieser Mechanismus eine sehr breite Wirtsabwehr dar. Als eine Form der unspezifischen Immunabwehr markiert PF4 Bakterien und stellt somit die Brücke zur spezifischen Immunabwehr her, die mit der Bildung von Antikörpern durch B-Zellen reagiert. Schließlich kann die Beseitigung der Bakterien Fc-Rezeptor abhängig durch Phagozyten, die wiederum zur unspezifischen Immunabwehr zählen, erfolgen. Dieser Immunmechanismus könnte einen Übergangstatus von dem evolutionär älteren unspezifischen zu dem evolutionär jüngeren spezifischen Abwehrsystem darstellen. Bekräftigt wird diese Hypothese durch erste Untersuchungen unserer Arbeitsgruppe, die zeigen, dass PF4 bereits in sehr primitiven Organismen vorkommt und hoch konserviert ist. Damit wird auch deutlich, dass die Funktion von PF4 wichtiger sein muss als bisher angenommen.

Bei der Therapie mit dem Polyanion Heparin wird dieser Mechanismus offensichtlich fehlgeleitet. Heparin bindet an Thrombozyten [83-85]. Diese tragen dadurch stark negativ geladene Polyanionen auf ihrer Oberfläche, an die PF4 bindet. Damit kann es zur Ausbildung von PF4/Heparin-Komplexen auf der Thrombozytenoberfläche kommen. Dies führt zum Mimikry: PF4/Heparin-beladene Thrombozyten sehen für das Immunsystem aus, wie PF4-beladene Bakterien. Welche Rezeptoren, diese Komplexe erkennen und die Bildung der anti-PF4/Heparin-Antikörper induzieren ist derzeit noch unklar. Wenn die entsprechenden B-Zellen jedoch stimuliert werden und diese anti-PF4/Heparin-Antikörper bilden, binden diese über den Fab-Teil an die Thrombozyten und lösen durch die Kreuzvernetzung von Thrombozyten-Fc-Rezeptoren über den Fc-Teil deren Aktivierung aus. Schließlich resultiert eine massive Thrombingenerierung, die unter Beteiligung aktivierter Thrombozyten, Thrombozyten-Mikropartikel, Endothelzellen und Monozyten zur HIT führen kann.

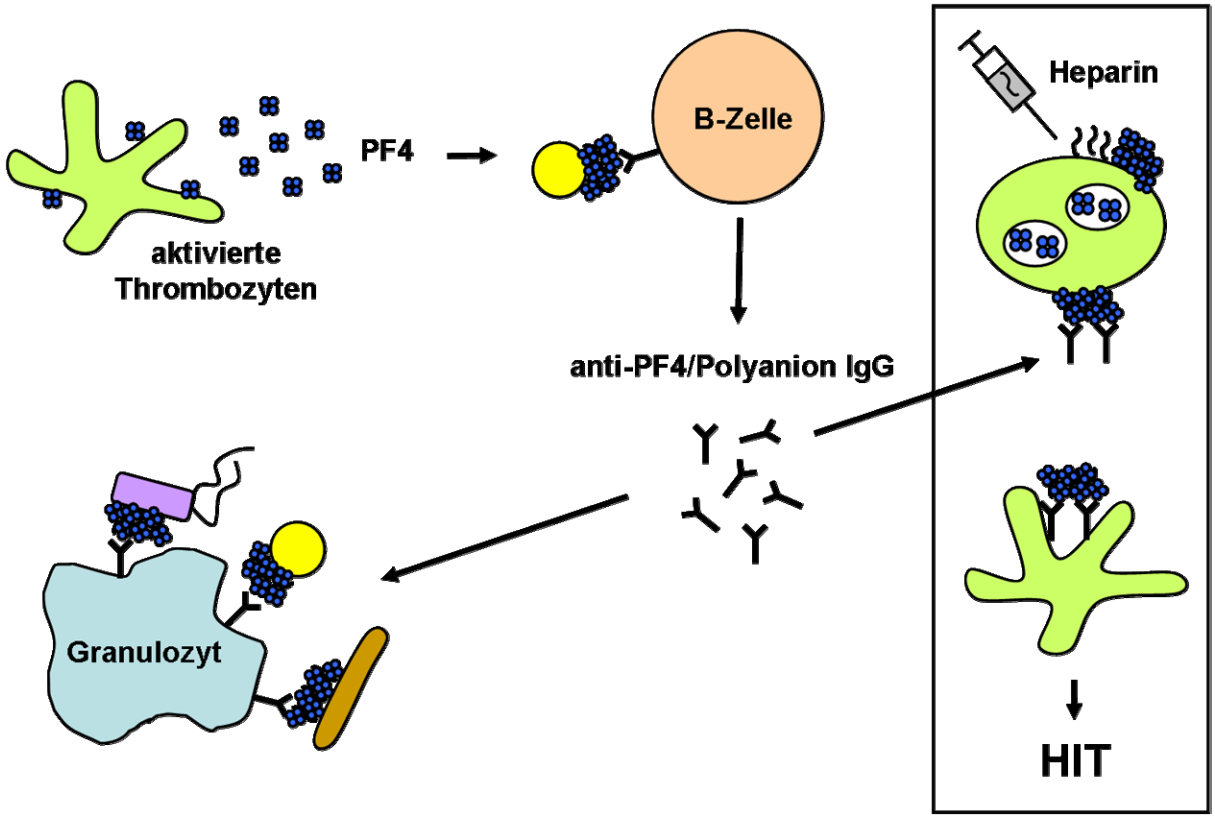

Abb. 27: Modell zur Vermittlung der breit reagierenden antibakteriellen Wirtsabwehr durch PF4, die bei Heparintherapie zur HIT führen kann.

Zusammenfassend betrachtet ergibt sich, dass die humorale IgG-Antwort auf an Bakterien gebundenem PF4 eine Wirtsabwehrstrategie darstellt, die bei Heparingabe unglücklicherweise den Nebeneffekt einer HIT bewirken kann. Die Ergebnisse sind von besonderer Bedeutung:

1. Sie erklären die bislang unklare Beobachtung, dass Patienten, die das erste Mal Heparin erhalten bereits an Tag 4-6 IgG-Antikörper gegen PF4/Heparin bilden.

2. Sie bieten eine pathophysiologische Erklärung für die derzeit häufigste immunvermittelte, unerwünschte Arzneimittelwirkung.

3. Die PF4-Bindung an Bakterien ist ladungsabhängig. Damit sind Antikörper gegen PF4/Polyanion-Komplexe nicht spezifisch für eine Bakterienspezies, sondern können eine Form der Wirtsabwehr darstellen, bei der eine IgG-Spezifität eine Vielzahl von Bakterien bindet.

3 Ausblick

Die vorliegende Dissertation bietet eine Reihe von Ansatzpunkten für neue Projekte.

Das wohl wichtigste Ergebnis dieser Arbeit ist, dass PF4 über die Bindung an eine Vielzahl unterschiedlicher Bakterienspezies eine sehr breit gefasste Wirtsabwehr vermitteln kann. Eine Antikörperspezifität kann nach Markierung der Bakterien mit PF4 eine Vielzahl von Bakterien binden und deren Phagoytose fördern. Erste Untersuchungen unserer Arbeitsgruppe zeigen, dass insbesondere die Heparinbindungsstelle des PF4-Moleküls in der Evolution hoch konserviert ist. Dies lässt vermuten, dass PF4 in der Evolution über die Bindung an Polyanionen auf der Bakterienoberfläche eine bedeutende Rolle in der Immunabwehr gespielt haben könnte. Bisher ist noch unklar, welche bakteriellen Polyanionen für die PF4-Bindung verantwortlich sind. Da PF4 positiv geladen ist und mit negativ geladenen Glykosaminoglykanen interagiert [64] liegt die Vermutung nahe, dass auch auf der Bakterienoberfläche negativ geladene Zuckerstrukturen eine Rolle spielen. In Frage kommen zum Beispiel Lipopolysaccharid (LPS) Gram-negativer Bakterien [61] und Teichonsäuren Gram-positiver Bakterien [60]. Zur Untersuchung der Interaktion dieser Substanzen mit PF4 könnte man von Bakterien aufgereinigtes LPS und Teichonsäuren zunächst im ELISA einsetzen. Auch gibt es Bakterienmutanten von *S. aureus*, die keine Wandteichonsäuren besitzen [86] bzw. Mutanten von *E. coli* [87] und *S. typhimurium* [88] mit sukzessive verkürzten LPS-Ketten. Diese Mutanten sollten eine verringerte PF4-Bindung in der hier vorgestellen durchflusszytometrischen Analyse im Vergleich zu den Wildtypen zeigen, wenn LPS und Teichonsäuren an der PF4-Bindung beteiligt sind.

Wie bereits erwähnt könnte das Maus-Sepsis-Modell CASP genutzt werden, um zugrunde liegenden Immunmechanismen hinsichtlich der Antikörperbildung gegen PF4/Polyanion-Komplexe aufzuklären. Dafür ließe sich das CASP-Modell an verschiedenen Knock-out-Mäusen anwenden. Eine mögliche T-Zell-unabhängige Entwicklung der Antikörper könnte mit T-Zell-Rezeptor-Knock-out-Mäusen aufgedeckt werden. Mit DEREG-Mäusen (DEpletion of REGulatory T cells), bei denen sich die Dämpfung der Immunantwort durch regulatorische T-Zellen gezielt ausschalten lässt, könnten die möglichen Regulationsmechanismen untersucht werden. Bei diesen Mäusen sind FoxP3-positive regulatorische T-Zellen über einen transgen koexprimierten Diphtherietoxin-Rezeptor *in vivo* depletierbar [89]. Schließlich wäre es auch interessant herauszufinden, welche B-Zellsubpopulation für die Produktion der anti-

PF4/Heparin-Antikörper verantwortlich ist. Potentielle Kandidaten wären Marginalzonen-B-Zellen. Dies sind B-Zellen mit autoreaktiven Eigenschaften, verantwortlich für die T-Zell-unabhängige humorale Immunantwort gegen Polysaccharid-Antigene und besonders empfänglich für die Aktivierung über „Toll-like"-Rezeptoren [90]. Da sie mit einer sehr schnellen Antikörperproduktion auf Pathogene in der Blutbahn reagieren können dienen sie der Überbrückung der frühen angeborenen und der später auftretenden adaptiven Immunität. Interessanterweise kommen sie noch nicht bei Neugeborenen vor [91], die nur äußerst selten eine HIT entwickeln [92]. Da sie in der Maus ausschließlich in der Milz lokalisiert sind [90] könnte man die Antikörperantwort nach CASP bei splenektomierten Mäusen untersuchen. Ein genehmigter Antrag des Tierversuchsvorhabens „Mechanismen zur Induktion von Antikörpern gegen PF4-Cluster in der murinen Sepsis anhand des Colon ascendens Stent-Peritonitis-Modells (CASP)" liegt bereits vor.

Ein weiterer offen gebliebener Punkt ist der Aufenthaltsort von PF4/Heparin IgG- und IgM-spezifischen B-Zellen, die das Vorkommen von anti-PF4/Heparin IgG und IgM bei gesunden Probanden erklären würden. Im Blut von Patienten nach kardiochirurgischer Operation, welche anti-PF4/Heparin IgG entwickelten, konnten keine anti-PF4/Heparin IgG produzierenden B-Zellen im Blut nachgewiesen werden [44]. Da man an dieser Stelle im humanen System aufgrund des begrenzten Probenzugangs nicht weiter kommt, wäre die Anwendung des PF4/Heparin-ELISPOTs (Enzyme-Linked Immuno Spot Technique), der für das humane System bereits in unserer Arbeitsgruppe etabliert wurde [44], mit präparierten B-Zellen aus verschiedenen lymphatischen Organen (Knochenmark, Milz, Lymphknoten) oder Peritoneal-Lavage der Maus denkbar. Zuvor müsste allerdings noch die Umstellung des ELISPOTs auf die Detektion muriner Antikörper produzierender B-Zellen erfolgen.

In dieser Arbeit wurde aufgezeigt, dass eine Vorimmunisierung mit PF4/Polyanion-Komplexen durch die Interaktion von PF4 mit Bakterien auftreten kann. Im Körper kommen aber auch physiologisch negative geladene Polyanionen wie Nukleinsäuren vor. Daher wäre denkbar, dass die Freisetzung von Desoxyribonukleinsäure (DNA) oder Ribonukleinsäure (RNA) bei Zelluntergängen eine Interaktion mit PF4 über die negativ geladenen Phosphatreste der Nukleinsäuren ermöglicht. Dadurch könnte es, ähnlich wie beim Heparin oder negativ geladenen Strukturen auf der Bakterienoberfläche, zur Ausbildung von Neoepitopen auf den aufgereihten PF4-Molekülen kommen. Somit könnte die Interaktion von PF4 und Nukleinsäuren einen alternativen Weg der Vorimmunisierung darstellen. Diese Hypothese ließe sich mit Untersuchungen zum Einfluss von Nukleinsäuren auf die PF4- oder

PF4/Heparin-Bindung an Thrombozyten und zur Kreuzreaktivität von anti-PF4/Heparin-Antikörpern gegen PF4/Nukleinsäure-Komplexe im ELISA oder im Funktionstest überprüfen.

Anhand der Ergebnisse dieser Arbeit wird jetzt verständlich, warum Patienten ohne vorherige Heparinexposition bereits sehr früh anti-PF4/Heparin-Antikörper der Klasse IgG bilden, aber sie können nicht erklären, warum nicht alle Patienten mit anti-PF4/Heparin-Antikörpern eine klinische HIT mit Thrombozytopenie und Thrombosen entwickeln [35]. Da bereits gezeigt wurde, dass Aktivierungstests mit gewaschenen Thrombozyten sensitiver sind als mit Plättchen-reichem Plasma [93] liegt die Hypothese nahe, dass Plasmafaktoren den Durchbruch einer klinischen HIT beeinflussen könnten. Mittels Methoden der Plasma-Fraktionierung und Massenspektrometrie könnten potentielle Kandidaten identifiziert werden und dann hinsichtlich ihres Einflusses auf entscheidende pathogene Reaktionen in der HIT, wie zum Beispiel die PF4/Heparin-Komplexbildung, weiter getestet werden. Dies beinhaltet das Potential einen neuen Risikofaktor für kardiovaskuläre Erkrankungen zu identifizieren.

Schließlich ließen sich die neuen Erkenntnisse hinsichtlich der Pathogenese der HIT möglicherweise auch auf andere immunvermittelte Erkrankungen anwenden.

4 Abkürzungsverzeichnis

A. a.	*Aggregatibacter actinomycetemcomitans*
aT	aktivierter Thrombozyt
BZ	B-Zelle
CASP	colon ascendens stent peritonitis
CD40- L	CD40-Ligand
ClfA	Clumping factor A
CR	Chemokinrezeptor
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DEREG	depletion of regulatory T cells
DNA	deoxyribonucleic acid
DZ	Dendritische Zelle
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immuno spot technique
EZ	Endothelzelle
Fbn	Fibrinogen
FITC	Fluoresceinisothyocyanat
FOXP3	forkhead box P3
FPA,B	Fibrinopeptid A, B
GMFI	geometric mean fluorescence intensity
HEK	human embryonal kidney
HIPA	Heparin-induzierte Plättchenaktivierung
HIT	Heparin-induzierte Thrombozytopenie

hu-PF4	humaner PF4
IgG	Immunglobulin
kDa	Kilodalton
L. monocytogenes	*Listeria monocytogenes*
Log	logarithmisch
LPS	Lipopolysaccharid
LTA	lipoteichoic acid
LZ	Leukozyt
mu-PF4	muriner PF4
NETs	neutrophil extracellular traps
N. meningitidis	*Neisseria meningitidis*
n.s.	nicht signifikant
OD	Optische Dichte
PAF	platelet-activating factor
PBP	platelet basic protein
PCS	photon correlation spectroscopy
PDGF	platelet-derived growth factor
PerCP-Cy5.5	Peridinin-Chlorophyll-Protein-Komplex-Cyanin-5.5
PF4	Plättchenfaktor 4
P. g.	*Porphyromonas gingivalis*
PMN	polymorphonuclear neutrophils
PSGL-1	P-Selektin Glykoprotein Ligand-1
RANTES	regulated upon activation normal T-cell expressed and secreted
RNA	ribonucleic acid
S. aureus	*Staphylococcus aureus*
SHIP	study of health in pomerania

SpA	staphylococcal protein A
S. pneumoniae	*Streptococcus pneumoniae*
T-ß-4	Thymosin-ß-4
ß-TG	ß-Thromboglobulin
TLR	toll-like receptor
TRIS	Tris(hydroxymethyl)-aminomethan
TZ	T-Zelle
UFH	unfraktioniertes Heparin
vWF	von Willebrand-Faktor

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6 Publikationen

6.1 Heparin-induced thrombocytopenia

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Unterschrift des Promovenden

Unterschrift des Betreuers

Heparin-induced thrombocytopenia

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Keywords
Heparin, heparin-induced thrombocytopenia, platelets, pathogenesis, immunology, anticoagulants

Summary
Heparin-induced thrombocytopenia (HIT), typically occurring in the second week of heparin therapy, is an antibody-mediated adverse drug reaction associated with increased thrombotic risk. The most important antigens are located on platelet factor 4 (PF4)/heparin complexes. HIT is always caused by platelet-activating antibodies, but not all PF4/heparin-reactive antibodies cause HIT. Thus, tests have a high negative, but only a moderate, positive predictive value. Clinical suspicion of HIT requires cessation of heparin and substitution with an alternative anticoagulant. As these drugs have an increased bleeding risk, they should be used in therapeutic doses only if HIT is considered very likely. Avoiding/postponing coumarin is crucial in minimizing microthrombotic complications. Recent studies of HIT immunobiology suggest that HIT mimics immunity against repetitive antigens, as are relevant in microbial defense. Thus, understanding HIT may help unravel why host defenses can trigger autoimmunity.

Schlüsselwörter
Heparin, Heparin-induzierte Thrombozytopenie, Thrombozyten, Pathogenese, Immunologie, Antikoagulanzien

Zusammenfassung
Die Heparin-induzierte Thrombozytopenie (HIT) ist eine Antikörper-vermittelte, unerwünschte Arzneimittelreaktion, die mit einem erhöhten Risiko für thromboembolische Komplikationen verbunden ist. Die HIT tritt typischerweise in der zweiten Woche der Heparingabe auf. Das wichtigste Antigen ist der Komplex zwischen dem Chemokin Plättchenfaktor 4 (PF4) und Heparin. Die HIT wird immer durch Thrombozyten-aktivierende Antikörper induziert, aber nicht alle PF4/Heparin-Antikörper induzieren eine HIT. Daher haben alle Laborteste einen hohen negativ-prädiktiven Wert, aber nur einen eingeschränkt positiv-prädiktiven Wert.
Der klinische Verdacht auf HIT erfordert die Beendigung der Heparingabe und die Antikoagulation mit alternativen Antikoagulanzien. Da diese oft mit einem erhöhten Blutungsrisiko verbunden sind, sollten sie nur dann in therapeutischer Dosierung gegeben werden, wenn die HIT eine sehr wahrscheinliche Ursache eines Abfalls der Thrombozytenwerte ist. Durch die Vermeidung von Vitamin-K-Antagonisten in der Akutphase der HIT kann das Risiko von mikrovaskulären Gefäßverschlüssen bei HIT wesentlich reduziert werden. Aktuelle Untersuchungen zur Pathophysiologie der HIT weisen darauf hin, dass die HIT durch Immunreaktionen gegen repetitive Antigenstrukturen verursacht wird, wie sie ansonsten vor allem für die Bakterienabwehr von Bedeutung sind. Das weitere Verständnis der Immunologie der HIT kann beitragen, den Zusammenhang zwischen bakteriellen bzw. viralen Infektionen und der Induktion von Autoimmunerkrankungen besser zu verstehen.

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Heparin-induzierte Thrombozytopenie
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Heparin-induced thrombocytopenia (HIT) is an immunologic adverse effect of heparin therapy. Antibody-mediated platelet activation and consequent thrombin generation result in a fundamental paradox:

> Despite thrombocytopenia induced by an anticoagulant, the major clinical effect in HIT is enhanced risk for venous and/or arterial thrombosis (22).

Although HIT is relatively rare, so many patients are exposed to heparin, that the absolute number of patients affected by HIT is likely one of the highest of all adverse drug effects (besides chemotherapy induced thrombocytopenia) and certainly the highest of all immune mediated, drug-induced blood cell disorders.

Prompt diagnosis and introduction of alternative non-heparin anticoagulants are important to prevent further complications in HIT. However, diagnosis can be problematic, as the two leading symptoms of HIT are not specific for HIT:

- thrombocytopenia and
- thrombosis.

Particularly in patient populations with a high incidence of thrombocytopenia (e. g. critically ill patients) clinical diagnosis of HIT based on the platelet count is difficult. Up to 50% of intensive care patients develop thrombocytopenia, usually caused by many other reasons but not by HIT (15, 61). Also the second major symptom, new thrombosis, can cause a diagnostic dilemma. This is because the adverse drug effect HIT causes exactly the complications which should be prevented by heparin, i. e. new thrombosis and the challenge is to discriminate "heparin failure" (= underdosage) from HIT induced thrombosis (81). Testing for PF4/heparin antibodies can help to identify those patients in whom thrombocytopenia may be caused by HIT. However, PF4/heparin antibodies are much more frequent than clinical HIT.

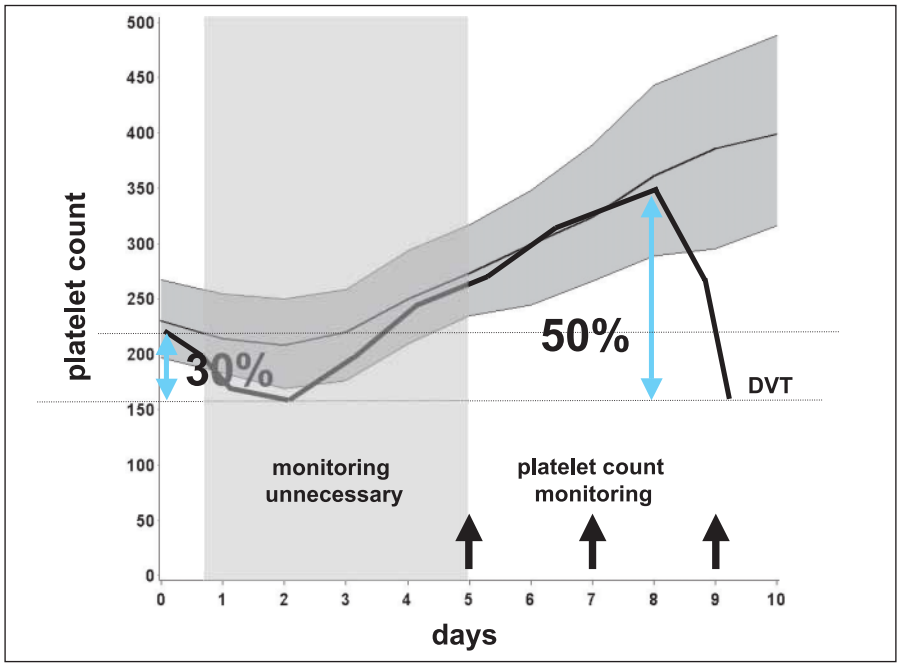

Fig. 1 Platelet count pattern with a reactive increase in platelet counts after trauma surgery. Reactive thrombocytosis following major surgery is very frequent and also generally considered to be a benign condition; however, it makes diagnosis of HIT difficult. The grey area shows the mean and the ±2 SD of the platelet counts in trauma patients. The solid black lines give the individual platelet count of a patient at day 9 developing HIT. For recognition of the 50% platelet count decrease which is one of the diagnostically leading symptoms of HIT it is mandatory to compare the actual platelet count with the platelet counts obtained between day 5 and 10. It is inappropriate to compare the actual platelet count with the baseline value before heparin, as this would cause underestimation of the platelet count decrease. HIT is based on immunological mechanisms. As anti-PF4/heparin antibodies do not reach clinically sufficient levels before day 5, it is sufficient to start monitoring of platelet counts from on day 5. As in the large majority of patients HIT manifests until day 12–14 and the platelet count peak is usually reached at day 10–12, platelet count monitoring at days 5, 7 and 9 are sufficient to recognize HIT.

Thus, the negative predictive value of PF4/heparin antibody assays is high, while the clinical relevance of the detection of anti-PF4/heparin antibodies strongly depends on the clinical context.

As a general rule this applies to all laboratory tests for HIT. The positive predictive value of PF4/heparin antibody tests can be increased by several laboratory maneuvers and especially by interpretation of assay results in the context of the clinical presentation of the patient (= in the context of the pretest probability). This approach using the Bayes' rule and the respective methodological background have been reviewed in detail elsewhere (71).

Clinical presentation

HIT usually features a platelet count fall >50% (from the highest value after day 4 of heparin treatment) and often new thrombosis, typically occurring 5–14 days after start of prophylactic or therapeutic dose heparin (5). Thromboembolic complications predominantly affect the venous system. Other rare complications include skin necrosis, adrenal haemorrhagic necrosis, or post-intravenous heparin bolus anaphylactoid reactions. The more unusual a new thrombosis during heparin treatment seems, the more HIT should be considered. HIT-associated thrombosis often occurs close to the 50% platelet count decrease and can even precede it by 1–2 days (~30% of thrombi) in a subset of patients (24). A platelet count fall within the first 4 days of heparin treatment is usually not HIT, unless the patient has been preimmunized and has circulating anti-PF4/heparin antibodies (46, 80). In these preimmunized patients the platelet count typically decreases within the first hours after start of heparin (= rapid onset HIT). Heparin-dependent antibodies tend to rapidly decrease in titers and become undetectable in >90% of patients within 100 days. In fact, in most patients, the heparin dependent, platelet activating antibodies disappear even much sooner, usually within one month.

Rapid onset HIT should be expected primarily in patients who did receive heparin within the preceeding 30 days.

This time kinetic of the PF4/heparin immune response has implications for platelet count monitoring during heparin therapy. A platelet count should be obtained before start of heparin (and in patients with recent heparin exposure also 12–24 hours thereafter to recognize rapid onset HIT). Monitoring of platelet counts at days 5, 7 and 9 is then sufficient to recognize HIT in the vast majority of patients (33).

Especially in surgical patients it is not appropriate to compare the platelet count value at the time of suspicion of HIT with the baseline value, because surgical patients typically show a reactive increase in platelet counts with overshooting platelet counts in the second week after major surgery (▶Fig. 1). Comparison of the actual platelet count with the baseline value will underestimate the magnitude of the platelet count fall.

To make it even more complex, the term HIT by itself is somewhat misleading as severe thrombocytopenia is rare in HIT. Most patients have only moderately decreased platelet counts and they can present even with high normal counts, e. g. patients with myeloproliferative disorders (65).

The mean platelet count in patients with HIT is 60×10^9/l and ranges from 15 to 150×10^9/l in 90% of patients (72, 73).

Patients with platelet counts less than 15×10^9/l rarely have HIT. In these patients other reasons for the platelet count decrease are much more likely, e. g. other

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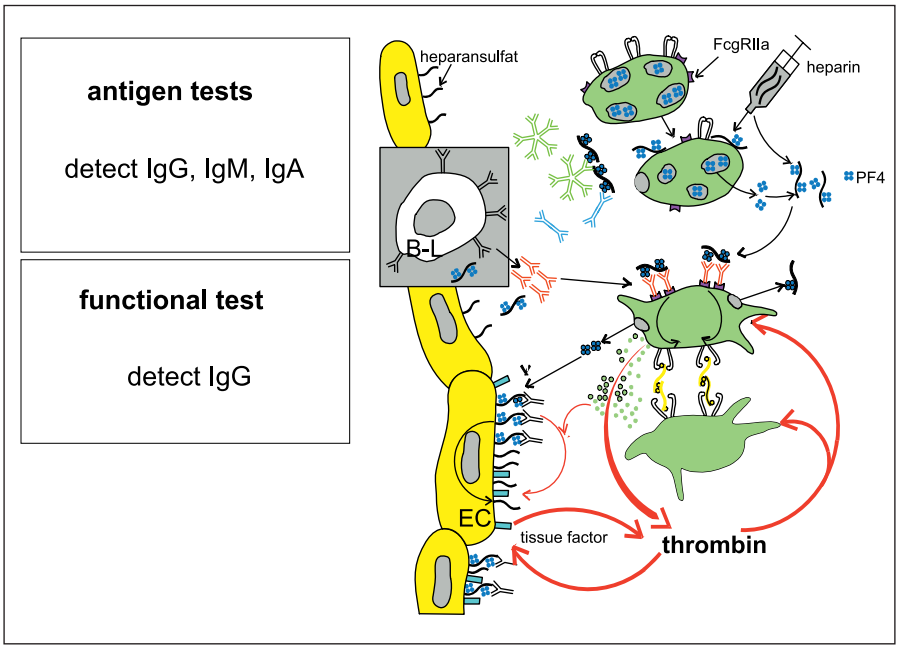

Fig. 2 Heparin binds to PF4. The resulting PF4/heparin complexes expose new epitopes and lead to formation of PF4/heparin antibodies. PF4/heparin complexes, as used in the antigen assays, are recognized by IgG, IgM, and IgA antibodies. However, only a subset of PF4/heparin IgG antibodies are able to bind to the platelet FcγIIa-receptor and activate platelets. This is measured by the functional assays. Thus functional assays provide the information about the platelet activating capacity of antibodies. As platelet activation is a prerequisite for enhanced thrombin generation and finally induction of thrombosis, the functional assays have a higher positive predictive value for HIT than the antigen assays.

drug-induced immune thrombocytopenias, GPIIb/IIIa inhibitor induced thrombocytopenia, or post-transfusion purpura (8, 25, 44).

Pathogenesis

Most frequently, HIT antigens are found on PF4 bound to heparin or other sulfated polysaccharides (4), forming linear multimolecular clusters (57). Immunogenicity (UFH > LMWH > fondaparinux) is influenced by

- relative size,
- amount, and
- stability of the PF4/heparin complexes.

Anti-PF4/heparin antibodies bind to PF4 via their F(ab) domains. The pathogenic immunoglobulin (Ig) isotype in clinical HIT is IgG. As serial PF4 molecules become aligned, several IgG molecules bind, leading to formation of large immune complexes that cross-link the platelet FcγIIa receptors (37). This triggers

- generation of platelet microparticles,
- activation of monocytes and endothelial cells, and finally
- activation of the clotting system, which results in massive thrombin generation.

Thus, thrombocytopenia does not result from reticuloendothelial system phagocytosis, but rather from intravascular platelet activation (14), with release of procoagulant platelet-derived microparticles (catalytic surface for enhanced thrombin generation). Only PF4/heparin IgG antibodies can bind and activate the platelet FcγIIa receptor (▶Fig. 2). This explains, why anti-PF4/heparin IgM and IgA antibodies have only a minor relevance in HIT as they have no Fc-part and cannot crosslink the FcγIIa receptor. The magnitude of thrombocytopenia correlates with thrombotic risk. Antibodies to other heparin-binding proteins (interleukin-8; neutrophil activating protein-2) have a minor role in HIT.

HIT antibodies also bind to endothelial cells, leading to procoagulant changes on microvascular endothelial cells (6). Further, HIT antibodies can activate monocytes (54) and neutrophils, forming platelet-monocyte-neutrophil aggregates. These pancellular activating effects could contribute to the prothrombotic nature of HIT.

PF4 complexes can be also be formed by other polyanions beside unfractionated heparin such as low molecular weight heparin (LMWH) or hypersulfated chondroitin-sulfate (32). In vitro also fondaparinux can induce complex formation of PF4 (26) and a few case reports have described HIT associated with fondaparinux treatment (58, 82). Furthermore, in some patients the B-cells producing the PF4/heparin specific antibodies start to produce antibodies with wider reactivity. The resulting antibodies then react also with PF4 bound to platelets in the absence of heparin. These antibodies can cause delayed onset HIT, which manifests several days after cessation of heparin (79). In rare cases, such antibodies can occur without exposure to heparin (70). These latter antibodies are true autoantibodies. They are usually present in very high titer and can persist for months.

Diagnosis of HIT

The 4-T Score

The frequency of HIT depends on the patient population(21,83). The typical features of HIT as described above have been summarized in a scoring system, which seems to be suitable for clinical application: see **www.medizin.uni-greifswald.de/transfus/**.

The 4-T score, validated by Lo et al. (43) (▶Tab. 1, ▶Fig. 3), gives points according to four clinical criteria: thrombocytopenia, timing, thrombosis and other reasons for thrombocytopenia. According to the score the clinical probability of HIT is

- low (0–3 points),
- intermediate (4–6 points), or
- high (7–8 points).

For patients with a low score of less than 4 points, the probability of having immune HIT is less than 5% and testing for PF4/heparin antibodies is not recommended. A laboratory test for PF4/heparin antibodies

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Tab. 1 Pretest scoring system for HIT: the 4 Ts (43)

4 Ts	2 points	1 point	0 points
Thrombocyto-penia	platelet count fall >50% and platelet nadir ≥20	platelet count fall 30–50% or platelet nadir 10–19	platelet count fall <30% or platelet nadir <10
Timing of platelet count fall	clear onset between days 5–10 or platelet fall ≤1 day (prior heparin exposure within 30 days)	consistent with days 5–10 fall, but not clear (e. g. missing platelet counts); onset after day 10 or fall ≤1 day (prior heparin exposure 30–100 days ago)	platelet count fall <4 days without recent exposure
Thrombosis or other sequelae	new thrombosis (confirmed); skin necrosis; acute systemic reaction postintravenous UFH bolus	progressive or recurrent thrombosis; non-necrotizing (erythematous) skin lesions; suspected thrombosis (not proven)	none
other causes for Thrombocytopenia	none apparent	possible	definite

UFH: unfractionated heparin

should be rather performed in patients with an intermediate score (4–6 points), while for patients with a high clinical probability (7–8 points) a direct switch of treatment to an alternative anticoagulant is recommended and laboratory testing should be performed in retrospect. However, the scoring system has to be applied carefully and sometimes requires "detective work" on the patient history.

Laboratory testing

Beside application of the scoring system, testing for PF4/heparin antibodies is the second most important measurement to exclude or to confirm the diagnosis of HIT in patients with clinically suspected HIT. Two groups of serological assays are available. The first group are the antigen tests. They consist of ELISAs, particle gel immunoassays or particle immunofiltration assays, which recognize binding of antibodies to PF4/polyanion complexes. The second group are the washed platelet assays, which detect heparin-dependent activation of platelets by the patient serum. The big advantage of the functional HIT assays is that they reflect in vitro the pathogenic cascade of HIT one step further downstream than the antigen tests. Thus they have a higher positive predictive value for HIT (Figure 2). In fact only about 50% of patients testing positive in the antigen tests also test positive in the functional assays (27).

PF4/heparin antigen assays

Solid phase ELISAs

Antigen assays detect the antibodies based upon binding to PF4/polyvinyl-sulfonate complexes or PF4/heparin complexes bound to a solid phase. Currently three commercial ELISAs are available: One from the Genetics testing institute (GTI, Waukesha, Wis), the Asserachrom PF4 (Diagnostica Stago, Parsippany, NJ) and the Zymunt-HIA (Hyphen BioMed, Neuville-sur-Oise, France). They detect anti-PF4/heparin antibodies of the IgG/IgA/IgM class, or IgG only, depending on the secondary antibodies used. These assays differ considerably in their structure. One ELISA uses PF4/polyvinyl-sulfonate complexes with PF4 purified from outdated platelets (GTI), the second uses recombinant PF4 complexed with heparin (Diagnostica Stago), and the third utilizes heparin-coated surfaces to which a platelet-leukocyte lysate is added allowing formation of PF4/heparin complexes but also of complexes of heparin with other chemokines such as IL-8 or NAP-2. These assays give slightly different results but show an overall acceptable concordance, especially with high titer PF4/heparin antibodies (17,59) (and unpublished observations).

Particle gel immunoassay

The Particle Gel Immunoassay (PaGIA, DiaMed, Biorad, Cressnier, Switzerland) uses PF4/heparin complexes bound to red, high-density polystyrene beads, which are agglutinated, if the patient's serum contains anti-PF4/heparin antibodies. The particle gel immunoassay does not distinguish between IgG, IgA, and IgM antibodies (17) and is at best semiquantitative. However, serial dilution of the patient serum enables to some extend assessment of the antibody titer (1). Its biggest advantage is its fast turn-around time of less than 15 min. However, it does produce false negative results in a few patients with high probability of HIT (53). Therefore, in case of high clinical suspicion, a negative result in this assay should prompt testing of patient serum in another test system.

In contrast to the assays described above, the Particle Immuno Filtration assay produces considerable false negative and false positive results compared to the ELISA (86), and seem to have a poor correlation with the 4Ts score (19). The manufacturer claimes to have improved test procedures after these publications. However, new data on the validation of this modified version are not available.

Improving the predictivity of antigen tests for HIT

Detection of IgG-antibodies only

IgA and IgM anti-PF4/heparin antibodies are unlikely to cause clinical HIT as outlined above. Modifying the antigen test to detect IgG antibodies only is one strategy to

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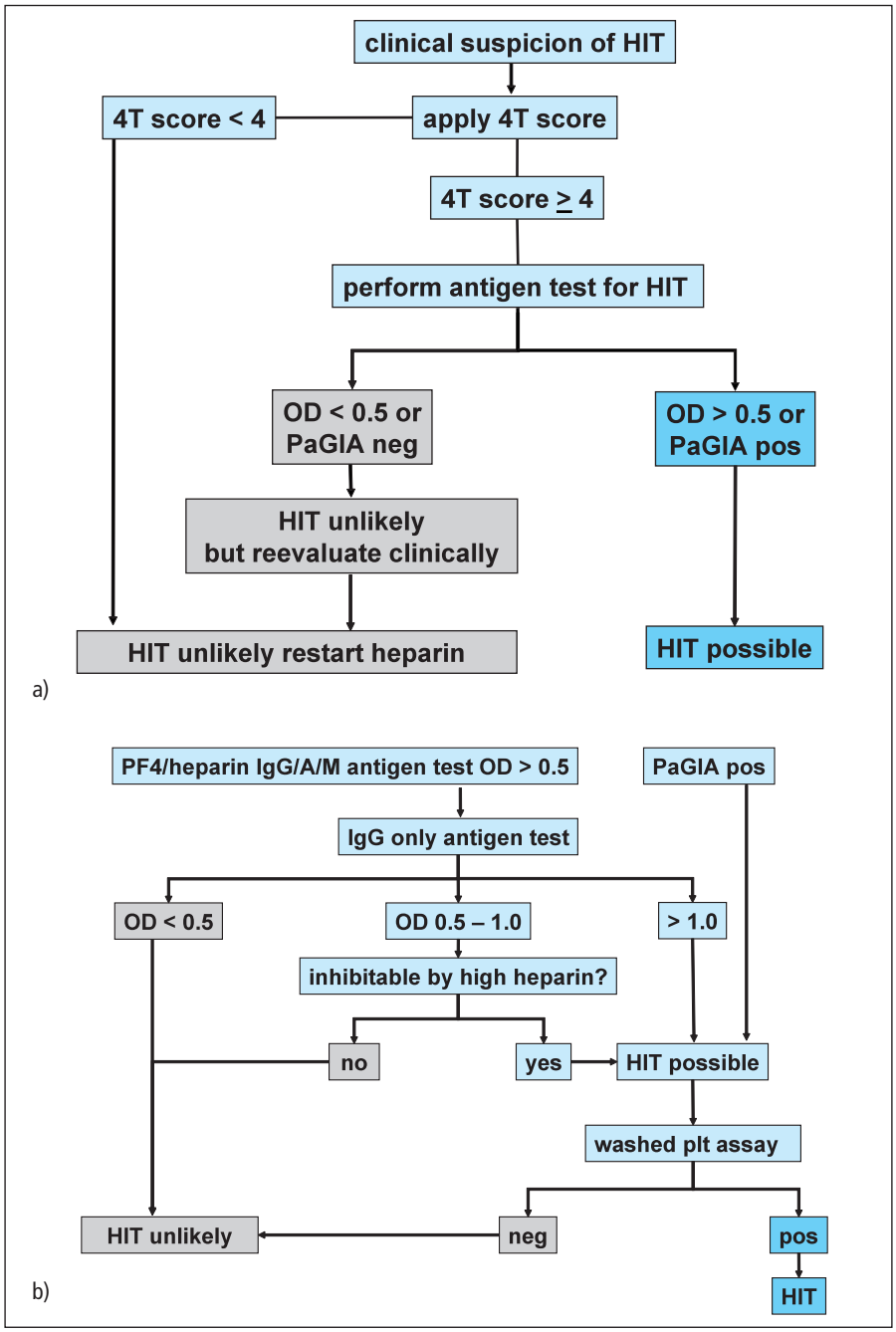

Fig. 3 A diagnostic algorithm for HIT:
1. Assess pretest probability for HIT (e.g., using the 4Ts). Patients with a low pretest probability (score ≤3) need no further testing and heparin can be maintained.
2. If the EIA is negative, HIT is very unlikely and heparin can be maintained. A positive result in a screening EIA indicates presence of anti-PF4/heparin antibodies. If an IgG EIA is weakly positive (OD<1.0), the antibodies are most likely non-platelet-activating. A confirmatory step using high heparin should be performed; if reactivity is not inhibited, HIT is very unlikely and heparin can be maintained.
3. An IgG EIA (OD > 1.0) indicates an increased risk for platelet-activating antibodies. These sera should ideally be assessed by a washed platelet activation assay. Demonstration of platelet-activating antibodies makes HIT very likely. A negative functional assay makes HIT unlikely and heparin can be maintained/restarted.
4. Clinical reassessment should support a final confirmation or exclusion of the diagnosis. However, it is a misconception to automatically retest patients whose EIA is negative (since the EIA is positive even during the earliest phase of HIT) (87).

increase specificity of the antigen tests for HIT(27, 41, 84).

Increasing the PF4/heparin ELISA cut-off

The probability of anti-PF4/heparin antibodies for being causative for the low platelet count or new thrombosis increases with the magnitude of the EIA OD result (35, 36, 59, 85, 88, 91). However, a subgroup of anti-PF4/heparin antibodies with platelet activating capacity show an OD < 1.0 in the PF4/heparin EIA, only (36). As higher the OD value is, as higher is the likelihood for anti-PF4/heparin IgG antibodies to be clinically relevant. However, there is no clear cut off to distinguish between clinically relevant and non-relevant antibodies.

Interpretation of ELISA test results according to a fixed OD cut off faces the problem that OD units are non-standardized arbitrary units. They might differ considerably between different photometers. This problem is similar to the issue of inter-laboratory comparison of the prothrombin time (or the Quick's value). As long as there is no standard for anti-PF4/heparin antibodies, the OD of the ELISA could be used as an estimate for the likelihood of a clinically relevant antibody, only.

Confirmatory step with high heparin

Beside antibodies with specificity to PF4/heparin antibodies, some individuals have also antibodies against PF4 alone present in their serum (52). These antibodies seem to be especially prevalent in patients with anti-phospholipid syndrome (47, 52). However, their clinical relevance is unresolved. These antibodies bind to PF4 but also to PF4 within the PF4/heparin complexes. The PF4/heparin complexes to which the pathogenic complex-specific antibodies bind, form only at a certain stoichiometric ratio of PF4 and heparin (23). High dose heparin disrupts the multimolecular PF4/heparin complexes (30) by saturating the heparin binding sites of all PF4 molecules. This has the potential to identify antibodies which are not specific for PF4/heparin but recognize PF4 alone (89), as these antibodies will still bind to

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non-complexed PF4. However, highly reactive sera may not be inhibited by high heparin, despite having platelet activating capacity and causing HIT in vivo (74). We assessed this systematically in 1000 consecutive samples of patients with suspected HIT and found that the high heparin inhibition step is helpful to differentiate between antibodies binding to PF4/heparin complexes (potentially pathogenic) and those binding to PF4 alone (clinical relevance unknown) as long as the reactivity of these antibodies is weak, as defined in our laboratory with an OD of < 1.0 (▶Fig. 3b). Sera of patients with higher reactivity in the ELISA seem to contain both, PF4/heparin complex antibodies and PF4-alone reactive antibodies. In about half of these sera a lack of inhibition by high heparin does not exclude relevant PF4/heparin antibodies (3).

Platelet activation assays

PRP or whole blood aggregation assays

Assays based on whole blood or platelet rich plasma of healthy donors are the least sensitive functional assays, which have a sensitivity of about 30% of the washed platelet assays discussed below. If a PRP or whole blood assay is positive, there is a high likelihood for the presence of clinically relevant antibodies. However, in critically ill patients, PRP based platelet activation tests sometimes give false positive results (76).

Washed platelet assays

Two assays are more widely used, the serotonin release test, which detects the release of radioactive serotonin from labelled donor platelets as a platelet activating endpoint (63) and the heparin-induced platelet activation assay (HIPA) (29), which detects platelet aggregation induced by patient serum. Both assays are performed on microtiter plates and allow for several quality controls. The most important is the two point approach, using low (0.1 to 0.3 IU/ml heparin) and high (100 IU/ml heparin) concentrations of heparin. Both tests are considered positive if a patient serum causes platelet activation at low but not at high heparin concentration. Other controls are the addition of hirudin to quench all effects of residual thrombin in the patient sample and the use of a monoclonal antibody, which blocks the platelet Fc-receptor, to demonstrate that platelet activation is caused by a heparin and FcγIIa receptor-dependent mechanism. Washed platelet assays have a higher predictive value for HIT compared to the antigen tests.

A diagnostic algorithm for HIT is given in ▶Figure 3.

Treatment

If there is high clinical suspicion for HIT, stopping heparin alone is insufficient. To prevent new thrombosis, non-heparin anticoagulant therapy is required. Vitamin K antagonists (VKA) must not be given in acute HIT. They can induce venous limb gangrene because of the early effects on protein C, they precipitates venous limb gangrene and/or skin necrosis in the extreme hypercoaguable milieu of HIT. Therefore vitamin K should be given if HIT is recognized only after VKA treatment has been started (78).

Three drugs are approved for anticoagulation in HIT:

- the two direct thrombin inhibitors (DTIs), lepirudin and argatroban, and
- the heparinoid danaparoid.

Rational therapies for HIT (78) are also

- the DTI bivalirudin, and
- the anti-factor Xa inhibitor fondaparinux.

All alternative anticoagulants confer significant risk for major bleeding (0.8–1.25% per treatment day) and no antidote is available. In only 5% of patients clinically suspected to have HIT, the diagnosis is confirmed (even fewer in ICU patients). Therefore, in patients with low/moderate clinical probability for HIT, our practice to reduce the risk of bleeding is to use prophylactic dose alternative anticoagulation, pending results of laboratory testing.

Direct thrombin inhibitors

DTIs are usually monitored by aPTT. However, a major problem is inappropriate dose reduction, when low prothrombin levels (e.g., because of VKA therapy) result in falsely elevated aPTTs during DTI treatment (42). This often results in catastrophic outcomes. The ecarin chromogenic assay overcomes this issue by providing a linear dose-response curve for DTIs independent of prothrombin levels.

Lepirudin (recombinant, bivalent, irreversible DTI; $t_{1/2}$ ~90 min) approved dosing is too high; recommended dosing must be adjusted downwards even further in patients with renal dysfunction (45, 78). Lepirudin induces antibodies in about 40–70% (re-exposure) of patients, which may reduce elimination, thereby prolonging lepirudin half-life. IgG-mediated anaphylactic reactions are rare and avoidable by omitting the bolus.

Argatroban (synthetic, monovalent, reversible DTI, $t_{1/2}$ ~45 min) substantially increases the INR, complicating argatroban-VKA overlap. While cessation of DTIs before measuring INR and restart DTIs if the INR is not in the therapeutic range avoids major bleedings (9), the patient is at increased risk for thrombosis during this phase. Functional clotting assays cannot be interpreted readily during argatroban treatment (40). In patients with reduced liver perfusion, 75–90% dose reduction to 0.2–0.5 µg/kg/min is important (11).

Bivalirudin (synthetic, bivalent, reversible DTI, $t_{1/2}$ ~25 min) (77) is cleaved by thrombin, which avoids major drug accumulation; the dose still needs ~50% reduction in patients with renal and/or hepatic dysfunction. Experience with bivalirudin in HIT is anecdotal(20, 38).

Indirect FXa inhibitors

Danaparoid is an AT-dependent heparinoid (13) with predominant anti-factor Xa activity ($t_{1/2}$ ~24 hours). The dose-response relationship is predictable, thus monitoring of therapeutic-dose treatment (anti-FXa activity) is required in patients with severely impaired renal function. In vitro cross-reactivity with HIT antibodies is of minor clinical relevance. Therapeutic dosing is indicated when HIT is strongly suspected or confirmed, with prophylactic dosing appropriate when suspicion for HIT is moderate.

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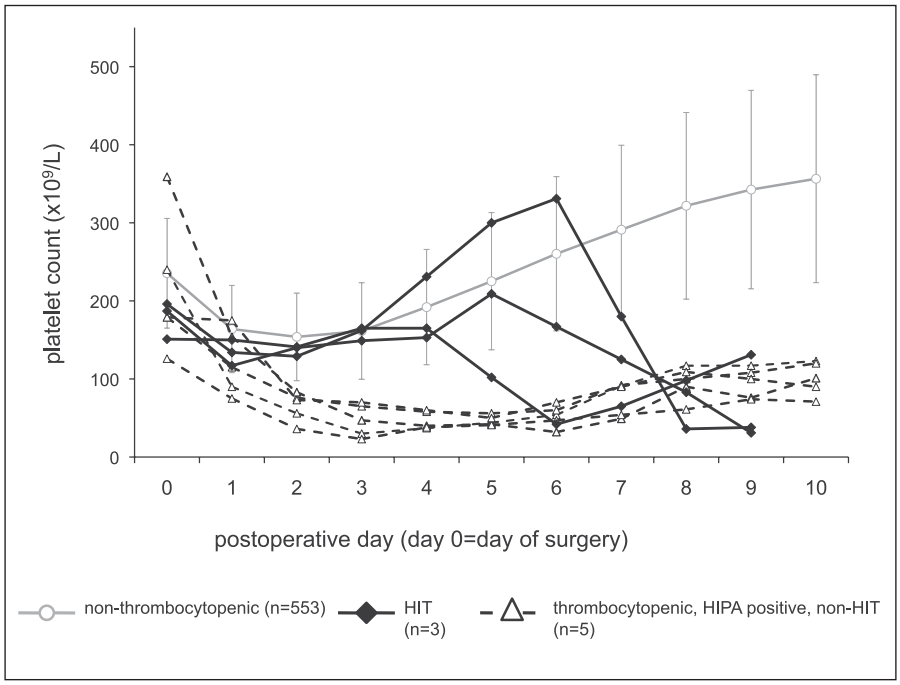

Fig. 4 Platelet counts before and after cardiac surgery of 553 patients without significant pathological platelet count changes are shown with ±1 standard deviation (SD) as white circles. Three patients with a platelet count fall after day 5, and who were adjudicated as having HIT, are presented with black rhombi and continuous lines. The grey triangles with continuous lines indicate mean platelet counts of 5 patients with early-onset and persisting thrombocytopenia and being HIPA-positive, but adjudicated as not having HIT (61).

Fondaparinux (AT-dependent FXa inhibitor; $t_{1/2}$ ~17 hours, considerably prolonged in patients with renal impairment) may be used in patients with low or moderate suspicion of HIT. Experience in treatment of acute HIT with thrombosis with therapeutic-dose fondaparinux is anecdotal (16).

Phenprocoumon

Most limb amputations in HIT result from microthrombosis due to severe DIC or (more often) VKA therapy. Thus, avoiding/postponing VKA use until platelet count recovery is a fundamental for HIT management. Inappropriate DTI dose reductions/cessation due to VKA-related aPTT prolongation is the rationale for giving vitamin K when HIT is recognized after VKA use (78).

New concepts of HIT

Special patient groups

The problem of HIT-antibody testing in cardiac surgery patients

Interpretation of a positive anti-PF4/heparin test is especially problematic in cardiac surgery patients. Up to 70% of these patients (depending on the test performed) test positive for anti-PF4/heparin antibodies during the first 10 days post-cardiac surgery, with only about 0.5–2% of patients presenting with clinically-evident HIT (55, 61, 68).

The problem of PF4/heparin antibody testing even starts before surgery. It has been a challenging observation that already before cardiac surgery anti-PF4/heparin antibodies are present in up to 20% of these patients (10, 12, 18, 50). However, the biological role of these antibodies is unclear. Particularly, it is controversial whether the presence of these antibodies predicts for adverse outcomes (18, 39, 50). We prospectively screened 591 patients before cardiac surgery for heparin-dependent antibodies by a PF4/heparin immunoassay (EIA) and platelet-activation test, and followed these patients for 30 days and found no evidence for an association of adverse outcomes post surgery and the presurgery anti-PF4/heparin IgG antibody status (60). Thus we strongly discourage testing for PF4/heparin antibodies in otherwise asymptomatic patients before cardiac surgery.

It is well accepted that the platelet count decrease occurring directly after cardiac surgery (due to hemodilution, platelet loss and consumption) (51) is not indicative for HIT, but rather if a patient develops a second episode of thrombocytopenia beginning within a narrow 'window' between postoperative days 5 and 10 (56). However, there is an additional group of patients, in whom it is very difficult to exclude HIT. These are those patients, in whom the early-onset thrombocytopenia after cardiac surgery persists beyond the first five days and thus extends into the period in which HIT typically occurs. When these patients test positive for anti-PF4/heparin antibodies, the crucial issue is whether the patient has developed 'true' HIT or whether a preceding – and persisting – surgery-related non-HIT thrombocytopenic disorder is simply associated with incidental and clinically-irrelevant seroconversion (75). We systematically assessed this question in a prospective study and found early-onset and persisting thrombocytopenia in 4.3% of patients after cardiac surgery. 5 of these 25 patients tested positive for PF4/heparin antibodies and also positive in the HIPA test. But none of the 5 patients developed thrombosis and in none of the 4 patients who switched to a non-heparin anticoagulant did the platelet counts subsequently recover by day 10 (▶Fig. 4). Thus a causal role of platelet-activating antibodies for thrombocytopenia appeared very unlikely (61). This provides a rationale for considering continued heparin therapy in such patients even if a PF4/heparin antibody test is positive. Maintaining heparin should be particularly considered when there is a reasonable alternative explanation for the thrombocytopenia and there is no new thrombosis (which would increase the likelihood of 'true' HIT), and no additional

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platelet count decrease of at least 30% occurs during the characteristic HIT 'window' between days 5 and 10 (62).

Chronic renal replacement therapy

Patients under chronic renal replacement therapy test positive for anti-PF4/heparin antibodies in 0.26 to 10% (7, 34). However, in most studies these antibodies were not an independent risk factor for arterial cardiovascular events, venous thromboembolism, vascular access occlusion, or mortality (31). However, in a study of Matsuo T et al. (48) these antibodies predicted for adverse clinical outcomes. Based on our own experience three risk groups of patients have to be differentiated:

1. Patients in whom renal dialysis is started. These patients are at a considerable risk to develop HIT during the first 2–3 weeks of dialysis and new thrombotic complications in the context of a positive functional HIT assay should raise a high suspicion for HIT.
2. Patients under chronic hemodialysis. In those patients anti-PF4/heparin antibodies and heparin dependent platelet activating antibodies are usually an epiphenomenon with little, if any clinical relevance.
3. Patients under chronic renal replacement therapy, who undergo a surgical procedure. These patients are again at an increased risk to develop HIT within the next 2 weeks after surgery (67). It seems that the immune system of these patients is altered by the surgical intervention in a way that HIT becomes more likely.

A very interesting observation is the successful re-exposure of a dialysis patient with HIT to heparin. This patient had been treated with an alternative anticoagulant until the PF4/heparin antibodies disappeared and then received heparin again during dialysis without adverse effects (49). Preliminary data imply that this approach might be feasible in other patients, too (69).

Anti-phospholipid syndrome

The antibody-mediated disorders HIT and antiphospholipid antibody syndrome (APS) have remarkably similar clinical presentations. Both are associated with thrombocytopenia and a high risk of thrombosis. It is likely that HIT is overdiagnosed in APS (47) as about 70% of APS patients have anti-PF4-antibodies, which can cause false positive PF4/heparin antigen tests. Very recently, Sikara et al (64) reported that the main antigen in anti-phospholipid syndrome, β2-Glykoprotein I, forms complexes with PF4. It might be that this protein-protein interaction requires new interpretation of test results in antiphospholipid syndrome as well as in HIT, but this requires further studies.

Immunology of HIT

Currently, the most interesting aspect of HIT is its immunobiology. HIT neither exhibits features typical of a primary immune response (initial formation of IgM antibodies followed by a more delayed IgG response) nor the serological features of a secondary immune response (stronger and more persistent formation of IgG antibodies) (28). Most HIT patients form IgG antibodies between days 4–10, even with first heparin use. HIT antibodies do not persist, however (80). This profile seems more compatible with a non-T-cell-dependent B-cell response, as described for immune reactions against viral antigens with repetitive epitopes (90). Indeed, repetitive epitopes in HIT are expressed as structures with a distance of 4–6 nm contained within 100–150 nm size, linear, ridge-like clusters of PF4/heparin (26). This is within the range of repetitive viral epitopes found to cause T-cell independent B-cell activation. However, other arguments favor a T-cell-dependent immune reaction in HIT. T-cell-independent B-cell responses should be primarily IgM whereas in HIT IgG predominates, and in a mouse model the immune response against PF4/heparin was T-cell-dependent (66). This suggests there has been previous contact(s) between the immune system and the „HIT antigens".

Our current working hypothesis is that early exposure to PF4 complexes, perhaps induced by endogenous non-heparin factors, leads to PF4 clustering and a T-cell-dependent antibody class switch of B-cells. Later in life, these B-cells (possibly, marginal zone B-cells (2)) become again transiently activated when PF4 clusters are induced by heparin treatment, together with a proinflammatory milieu. This would explain why major surgery is a risk factor for HIT (through PF4 release and inflammation). At the GTH 2010 meeting Krauel K et al. present data showing that gram positive as well as gram negative bacteria bind PF4 in a charge dependent manner. PF4 forms complexes on the bacterial surface, thereby exposing the same epitopes which are formed on PF4/heparin complexes. It was even possible to purify platelet activating, heparin dependent antibodies from sera of patients with clinical HIT by adsorption and elution using PF4-coated bacteria. This strongly indicates that PF4/polyanion antibodies have a role in anti-bacterial host defense. During heparin treatment, heparin binds to platelets, which allows rebinding of PF4 and formation of PF4/heparin complexes on the platelet surface. Thus platelets mimick PF4-coated bacteria. Potentially HIT is a misdirected host defense mechanism. Understanding the immunobiology of HIT may help explain why host defenses can result in autoimmunity.

Conflict of interest

A. Greinacher has given lectures sponsored by: Organon Int, Schering-Plough, Essex Pharma (drug: danaparoid), Mitsubishi Pharma (drug: argatroban), Glaxo-Smith-Kline (drug: argatroban, fondaparinux), Pharmion (drug: lepirudin). He has served as a consultant for: Organon Int, Schering-Plough, Essex Pharma, Mitsubishi Pharma, Pharmion, Bayer, Novartis. He has received funding for research projects by Organon Int, Schering-Plough, Mitsubishi Pharma, Pharmion.

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6.2 Heparin-induced thrombocytopenia – therapeutic concentrations of danaparoid, unlike fondaparinux and direct thrombin inhibitors, inhibit formation of platelet factor 4-heparin complexes

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ORIGINAL ARTICLE

Heparin-induced thrombocytopenia – therapeutic concentrations of danaparoid, unlike fondaparinux and direct thrombin inhibitors, inhibit formation of platelet factor 4–heparin complexes

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Summary. *Background:* Treatment of heparin-induced thrombocytopenia (HIT), a disorder in which anti-platelet factor 4 (PF4)–heparin antibodies cause platelet activation and hypercoagulability, requires alternative (non-heparin) anticoagulation. Treatment options include direct thrombin inhibitors [lepirudin and argatroban (approved), and bivalirudin], danaparoid (approved) (mixture of anticoagulant glycosaminoglycans), or fondaparinux (synthetic heparin-mimicking pentasaccharide). PF4–heparin complexes form at optimal stoichiometric ratios. *Objectives:* To compare the effects of these various non-heparin anticoagulants in disrupting the formation of PF4–heparin complexes, and PF4-containing immune complexes. *Patients/methods:* Sera were obtained from patients with serologically confirmed HIT. The effects of the alternative anticoagulants on PF4 and PF4–heparin complex interactions with platelets, as well as HIT antibody binding and platelet activation, were investigated. *Results:* Danaparoid at very low concentrations increased PF4 binding to platelets. In therapeutic concentrations, however, it decreased PF4 binding to platelets ($P = 0.0004$), displaced PF4–heparin complexes from platelets ($P = 0.0033$) and PF4 from the surface of a PF4-transfected HEK-293 EBNA cell line expressing the PF4 receptor CXCR3-B ($P = 0.0408$), reduced PF4–heparin complex size ($P = 0.025$), inhibited HIT antibody binding to PF4–heparin complexes ($P = 0.001$), and prevented platelet activation by HIT antibodies ($P = 0.046$). Although fondaparinux also interfered with PF4 binding to platelets, HIT antibody binding to PF4–heparin complexes, and activation of platelets by HIT antibodies, these effects occurred only at supratherapeutic concentrations. The direct thrombin inhibitors had no effect at any concentrations. *Conclusions:* Danaparoid uniquely interferes with the pathogenesis of HIT by disrupting PF4-containing immune complexes at therapeutic dose concentrations. It is possible that these effects contribute to its therapeutic efficacy.

Keywords: danaparoid, direct thrombin inhibitors, fondaparinux, heparin, heparin-induced thrombocytopenia, platelet factor 4.

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Introduction

Heparin can cause the prothrombotic immune-mediated reaction, heparin-induced thrombocytopenia (HIT). HIT antibodies are primarily directed against complexes of platelet factor 4 (PF4) and heparin [1–3]. PF4 is a positively charged chemokine released from platelet α-granules upon platelet activation. At optimal molar ratios of PF4 and heparin – and in relation to heparin chain length and degree of sulfation [2,4] – multimolecular PF4–heparin complexes are formed. PF4–heparin complex formation leads to the expression of neoepitopes [5,6], most likely through close approximation of PF4 tetramers enabled by charge neutralization [7,8]. IgG antibodies against these complexes form PF4–heparin–IgG immune complexes, which activate platelets through FcγIIa receptors [3,9], producing hypercoagulability.

Patients with acute HIT usually require anticoagulation with an alternative agent. There are five anticoagulants with rationales for the treatment of HIT [10]. These include two

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antithrombin-dependent inhibitors with predominant (danaparoid [11]) or exclusive (fondaparinux [12,13]) anti-factor Xa inhibition, and three agents that are direct thrombin inhibitors (lepirudin [14], argatroban [15], and bivalirudin [16]). Danaparoid, lepirudin and argatroban are approved in this indication.

Danaparoid comprises a mixture of low molecular weight non-heparin glycosaminoglycans (GAGs) [17]. Its specific activity is relatively low (20 anti-FXa U mg^{-1}), reflecting the presence of high concentrations of non-anticoagulant GAGs among the minority of molecules that have anticoagulant activity. Thus, relatively high doses of danaparoid (in relation to anti-FXa effect) are required. In contrast, the pentasaccharide fondaparinux has very high specific activity (700 anti-FXa U mg^{-1}), reflecting its composition of uniformly active molecules. By comparison, unfractionated heparin (UFH) has intermediate specific activity ($\sim$ 150 anti-FXa U mg^{-1}), and the proportion of molecules with anticoagulant activity (approximately one-third) is also intermediate between those of danaparoid and fondaparinux.

Both danaparoid and fondaparinux interact with PF4. Whereas danaparoid sometimes increases platelet activation *in vitro* in the presence of HIT antibodies ('*in vitro* cross-reactivity') [18,19], it has also been reported to inhibit platelet activation by HIT antibodies [19,20]. Although fondaparinux only rarely exhibits *in vitro* cross-reactivity [21,22], its use has been associated with formation of anti-PF4–heparin antibodies [23,24]. Furthermore, two possible cases of HIT linked to preceding fondaparinux therapy have been reported [25,26]. Formation of complexes between PF4 and negatively charged polysaccharides is charge-dependent [19,27] and requires optimal ratios of both molecules [8,28].

In this study, we assessed systematically whether danaparoid and fondaparinux could influence HIT pathogenesis by interfering with PF4–platelet interactions and anti-PF4–heparin antibody binding. We also assessed the direct thrombin inhibitors as controls to exclude indirect, thrombin-related effects. Our study indicates that at clinically relevant (pharmacologic) concentrations, danaparoid differs considerably from these other anticoagulants, as it directly interferes with both PF4 and PF4–heparin complex binding to the platelet surface. Additionally, danaparoid disrupts pre-existing PF4–heparin complexes.

Materials and methods

Preparation of platelets

Ten milliliters of whole blood from healthy volunteers was anticoagulated with 10 μg mL^{-1} (final) hirudin [lepirudin (Refludan); Pharmion, Hamburg, Germany]. Platelet-rich plasma (PRP) was prepared by centrifugation (120 × *g*, 20 min, 30 °C). To obtain gel-filtered platelets (GFPs), up to 4 mL PRP was applied to a Sepharose CL-2B liquid chromatography column (30 mL; Sigma-Aldrich, Taufkirchen, Germany) pre-equilibrated with buffer [137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2.6H_2O$, 2 mM $CaCl_2.2H_2O$, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 0.4% bovine serum albumin (BSA), 0.1% glucose, pH 7.2].

Patient sera were collected from five patients with clinically suspected HIT (available 4T's scores are > 5 points) plus very strong antibodies (> 80% serotonin release) in all cases. Control sera were obtained from healthy donors.

All participants gave their written informed consent.

Influence of different anticoagulants on PF4 binding to platelets

GFPs (40 000 μL^{-1}) were incubated (30 min, 37 °C) with 25 μg mL^{-1} PF4 (ChromaTec, Greifswald, Germany) in the presence of increasing concentrations of anticoagulants or buffer: UFH, 0.01, 0.02, 0.04, 0.08, 0.16, 0.31, 0.63, 1.25, 2.5 and 5 IU mL^{-1}, 150 IU mg^{-1} (Braun, Melsungen, Germany); danaparoid, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64 and 1.28 anti-FXa U mL^{-1}, specific activity 20 anti-FXa U mg^{-1} (Orgaran; Organon, Munich, Germany); and fondaparinux, 0.007, 0.07, 0.35, 0.7, 3.5, 7, 35 and 70 anti-FXa U mL^{-1}, specific activity 700 anti-FXa U mg^{-1} (Arixtra, GlaxoSmithKline, London, UK). In a second series of experiments, the amount of PF4 added to the GFPs was also modified (5, 15, 25 and 35 μg mL^{-1}), before addition of increasing concentrations of danaparoid. After incubation with anticoagulant/buffer, the GFPs were fixed (1% paraformaldehyde; Merck, Darmstadt, Germany; 20 min, 4 °C), washed twice with 2 mL of buffer (600 × *g*, 7 min, 4 °C), incubated with rabbit anti-human PF4 (Dianova, Marl, Germany), fluorescein isothiocyanate (FITC)-labeled with the FluoReporter FITC Protein Labeling Kit (Molecular Probes, Eugene, OR, USA), and mouse anti-human CD42a–Phycoerythrin (PE) (BD Biosciences, San Jose, CA, USA), or isotype controls (30 min, 4 °C), washed, and analyzed by flow cytometry. Antibody binding was quantified by geometric mean fluorescence intensity (MFI).

Influence of different anticoagulants on PF4–heparin complex binding to platelets

In acute HIT, PF4–heparin complexes have already formed. We therefore tested the effects of different anticoagulants on GFPs (40 000 μL^{-1}) preloaded with PF4–heparin complexes (25 μg mL^{-1} PF4 and 0.08 IU mL^{-1} UFH, 30 min, 37 °C), allowing binding of PF4–heparin complexes at experimentally determined optimal concentrations. Then, platelets with bound PF4–heparin complexes were washed (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 0.4% BSA, 0.1% glucose, 2.5 U mL^{-1} apyrase, 0.1 μg mL^{-1} hirudin, pH 6.3; 600 × *g*, 7 min, 37 °C) to remove unbound PF4 and heparin, incubated with UFH (5 IU mL^{-1}), danaparoid, or fondaparinux (at the concentrations given above), or 10 μg mL^{-1} hirudin, argatroban (Mitsubishi-Pharma, Düsseldorf, Germany), or bivalirudin (The Medicines Company, Parsippany, NJ, USA), fixed, and processed as described above, before flow cytometric analysis.

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Influence of different anticoagulants on a PF4-transfected HEK-293 EBNA cell line

An HEK-293 EBNA cell line transfected with, and expressing, human PF4 and the PF4 receptor [29], kindly provided by P. Romagnani (Florence, Italy), was cultured in Dulbeccos' modified Eagle's medium (Invitrogen, Karlsruhe, Germany) and 10% fetal bovine serum until reaching confluence, harvested, adjusted to 3×10^6 mL^{-1} with buffer (phosphate-buffered saline + Ca^{2+}/Mg^{2+}, 0.2% BSA, pH 7.2), incubated with UFH, danaparoid, fondaparinux, hirudin, argatroban, bivalirudin (at the concentrations given above), or buffer (30 min, 37 °C), washed with 3 mL of buffer ($190 \times g$, 10 min, room temperature), incubated with rabbit anti-human PF4–FITC (30 min, 37 °C), and washed, before flow cytometric analysis.

Solid-phase PF4–heparin enzyme immunoassay (EIA)

An in-house EIA [30] that detects PF4–heparin IgG antibodies was modified by the addition of various anticoagulants to the HIT serum prior to incubation with PF4–heparin. In brief, the wells of a microtiter plate were coated with PF4–heparin complexes and incubated with HIT serum in the presence of either danaparoid (0.08, 0.16, 0.32, 0.64, 1.28, 2.56, 5.12 and 10.24 anti-FXa U mL^{-1}), fondaparinux (0.07, 0.35, 0.7, 7, 70, 350, 525 and 700 anti-FXa U mL^{-1}), 10 μg mL^{-1} hirudin, argatroban, or buffer, washed, incubated with alkaline phosphatase-conjugated goat anti-human IgG, and washed; *p*-nitrophenyl phosphate in 1 N diethanolamine buffer was then added. Five sera of patients with serologically confirmed HIT were tested.

Fluid-phase PF4–heparin EIA

This assay was modified from Newman *et al.* [18]. PF4 was biotinylated while heparin-binding sites were protected by binding PF4 to heparin Sepharose beads before incubating with biotin (Roche Diagnostics, Laval, QC, Canada). The functionality of biotinylated PF4 was confirmed by its successful use in the EIA. Diluted HIT sera, tested individually ($n = 5$), were incubated with PF4 (30% biotinylated) mixed with an optimal concentration of UFH in the presence of danaparoid, fondaparinux, hirudin, argatroban (at the concentrations given above), or buffer. This mixture was then added to protein G–Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) in a microcentrifuge tube. Biotin–PF4–UFH–IgG complexes were immunoprecipitated by centrifugation. The Sepharose was washed and then incubated with streptavidin–horseradish peroxidase (Amersham Pharmacia Biotech). After washing, K-Blue Max Substrate (Neogen Corporation, Lexington, KY, USA) was added. The Sepharose was centrifuged, and the reaction was stopped by adding 1 M HCl. Samples that produced optical density (OD) readings greater than or equal to the mean + 3 SD of 20 normals were considered to be positive.

Serotonin-release assay (SRA)

The ^{14}C SRA was performed as previously described [31] with 0.2 IU mL^{-1} UFH in the presence of either danaparoid, fondaparinux, hirudin, argatroban (at the concentrations given above) or buffer added to the diluted HIT serum ($n = 5$).

Photon correlation spectroscopy (PCS)

PF4 (20 μg mL^{-1}; filtered with a 0.22-μm membrane filter; MillexGP; Millipore, Cork, Ireland) was incubated with buffer (0.05 M NaH_2PO_4, 2% NaN_3, pH 7.5) or UFH (0.5 IU mL^{-1}; 30 min, room temperature). Then, danaparoid (0.1, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, 2.1, 5 and 10 anti-FXa U mL^{-1}), 10 μg mL^{-1} hirudin, argatroban or bivalirudin (60 min, 37 °C) was added, and the size of complexes was determined by PCS (Zetasizer 3000HS; Malvern Instruments, Malvern, UK) as mean hydrodynamic diameter (MHD), as previously described [7].

Statistical analysis

Comparison between groups was performed by paired-samples *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

Influence of different anticoagulants on PF4 binding to platelets

Baseline platelet surface PF4 levels (studies performed without preincubating platelets with PF4) were very low (not shown). After preincubation, PF4 bound to platelets, reaching maximal binding at 25 μg mL^{-1}. PF4 was then titrated in the presence of different concentrations of danaparoid, and again, maximum binding was seen at 25 μg mL^{-1} (Fig. 1A). Therefore, 25 μg mL^{-1} PF4 was used for further experiments, and the corresponding bound PF4 levels were arbitrarily set as 100% (corresponding to 85.3 MFI). UFH at the concentrations used for prophylactic-dose anticoagulation (0.16 IU mL^{-1} = 1.04 μg mL^{-1}) increased PF4 binding to platelets from 100% (baseline) to 265% ($P < 0.0001$); UFH at 0.31 IU mL^{-1} (= 2.1 μg mL^{-1}) increased PF4 binding 2-fold ($P = 0.0002$); however, UFH at higher concentrations (0.63 IU mL^{-1} = 4.17 μg mL^{-1}) started to decrease PF4 binding (– 30%; $P = 0.0029$) (Fig. 1B). Supratherapeutic concentrations of UFH (2.5 IU mL^{-1} = 16.67 μg mL^{-1}) decreased PF4 binding to platelets by > 95% ($P < 0.0001$). In contrast, danaparoid at therapeutic-dose concentrations (0.64 anti-FXa U mL^{-1} = 32 μg mL^{-1}) decreased PF4 binding to platelets by 83% ($P = 0.0004$), and at prophylactic-dose concentrations (0.16 anti-FXa U mL^{-1} = 8 μg mL^{-1}) it reduced binding by 75% ($P = 0.0023$). Interestingly, danaparoid also increased PF4 binding at concentrations below the range used for thromboprophylaxis (maximum at 0.04 anti-FXa U mL^{-1} = 2 μg mL^{-1}; $P = 0.0177$). However, this effect was much weaker than that seen with UFH (Fig. 1A,B).

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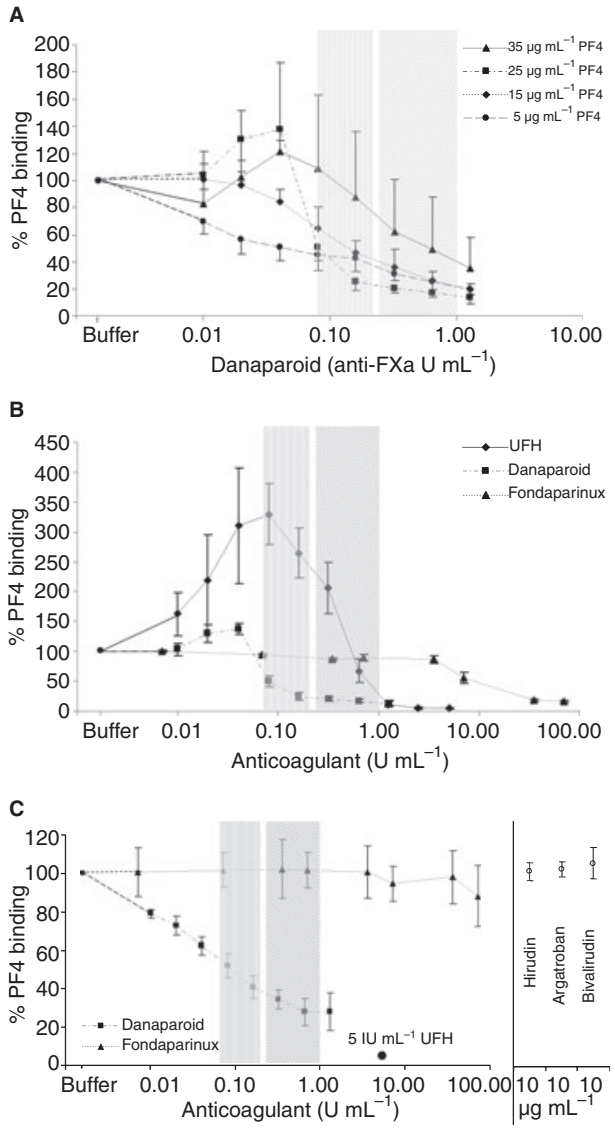

Fig. 1. Concentration dependent effects of different anticoagulants on PF4 binding to platelets. (A) Gel-filtered platelets (GFPs) were incubated with different concentrations of platelet factor 4 (PF4) (5, 15, 25 and 35 μg mL^{-1}) in the presence of increasing concentrations of danaparoid. Prophylactic-dose and therapeutic-dose concentration ranges of the anticoagulants are given as vertical and diagonal striped areas, respectively. PF4 binding in the presence of buffer was defined as 100%. Maximum PF4 binding appeared at 25 μg mL^{-1} PF4. Independently of the PF4 concentration, therapeutic-dose danaparoid inhibited PF4 binding to platelets. Data (mean ± SD) represent results of three experiments each. (B) GFPs were incubated with 25 μg mL^{-1} PF4 in the presence of increasing concentrations of unfractionated heparin (UFH), danaparoid, or fondaparinux. Prophylactic-dose and therapeutic-dose ranges of the anticoagulants are given as vertical and diagonal striped areas, respectively. PF4 binding in the presence of buffer was defined as 100%. Whereas prophylactic-dose and therapeutic-dose UFH increased PF4 binding to platelet surfaces, binding was decreased by supratherapeutic UFH concentrations (> 0.6 IU mL^{-1}). In contrast, danaparoid decreased PF4 binding at prophylactic and therapeutic concentrations, although an initial increase was observed at very low concentrations. Fondaparinux impaired PF4 binding only at supratherapeutic concentrations. Data (mean ± SD) represent results of eight (UFH) or three (danaparoid, fondaparinux) experiments. (C) GFPs preincubated with PF4–heparin complexes (and then washed) were incubated with increasing concentrations of danaparoid or fondaparinux, 5 IU mL^{-1} UFH, or 10 μg mL^{-1} hirudin, argatroban, or bivalirudin. Prophylactic and therapeutic concentration ranges are given as vertical and diagonal striped areas, respectively. PF4 binding after preincubation of platelets with PF4–heparin was defined as 100%. Prophylactic and therapeutic concentrations of danaparoid displaced PF4–heparin complexes from platelets. Fondaparinux at prophylactic and therapeutic levels, as well as hirudin, argatroban, and bivalirudin, had no effect. High concentrations of UFH served as control. Data (means ± SD) represent results of three (UFH, danaparoid), four (fondaparinux), or five (hirudin, argatroban, bivalirudin) experiments.

Fondaparinux neither increased nor decreased PF4 binding to platelets at clinically relevant concentrations (prophylactic dose, 0.07 anti-FXa U mL^{-1} = 0.1 μg mL^{-1}; therapeutic dose, 0.7 anti-FXa U mL^{-1} = 1 μg mL^{-1}) (Fig. 1B). However, at supratherapeutic concentrations (≥ 35 anti-FXa U mL^{-1} = 50 μg mL^{-1}), fondaparinux decreased PF4 binding to platelets by 81% (P = 0.0001).

Influence of different anticoagulants on PF4–heparin complex binding to platelets

Preincubation of platelets with PF4–heparin complexes (formed at optimal ratios) was defined as 100% binding (corresponding to 103.1 MFI). Danaparoid at prophylactic-dose concentrations (0.16 anti-FXa U mL^{-1} = 8 μg mL^{-1}) displaced PF4–heparin complexes by 59% (P = 0.0035), and at therapeutic-dose levels (0.64 anti-FXa U mL^{-1} = 32 μg mL^{-1}) it displaced PF4–heparin complexes by 72% (P = 0.0033) (Fig. 1C). Neither fondaparinux (in prophylactic or therapeutic doses) nor the direct thrombin inhibitors in supratherapeutic concentrations (10 μg mL^{-1}) exhibited any effect on PF4–heparin complex binding to platelets.

Influence of different anticoagulants on a PF4-transfected HEK-293 EBNA cell line

The signal of surface-bound PF4 without addition of any of the anticoagulants was set as 100% baseline (corresponding to 30.8 MFI). Danaparoid at prophylactic-dose concentrations (0.16 anti-FXa U mL^{-1} = 8 μg mL^{-1}) reduced PF4 binding to 53% (P = 0.0813) (Fig. 2). This inhibitory effect reached significance when therapeutic-dose concentrations (0.64 anti-FXa U mL^{-1} = 32 μg mL^{-1}) of danaparoid were used (59% inhibition; P = 0.0408). The other anticoagulants did not affect PF4 expression. High concentrations of UFH (5 IU mL^{-1} = 33.33 μg mL^{-1}) served as controls.

PF4–heparin EIA

We then assessed the impacts of the different anticoagulants on antibody binding to PF4–heparin complexes in a purified system. In the solid-phase EIA, danaparoid inhibited binding of anti-PF4–heparin antibodies to PF4–heparin complexes at prophylactic-dose concentrations (0.16 anti-FXa U mL^{-1} = 8 μg mL^{-1}; P = 0.005) and to a larger extent at therapeutic

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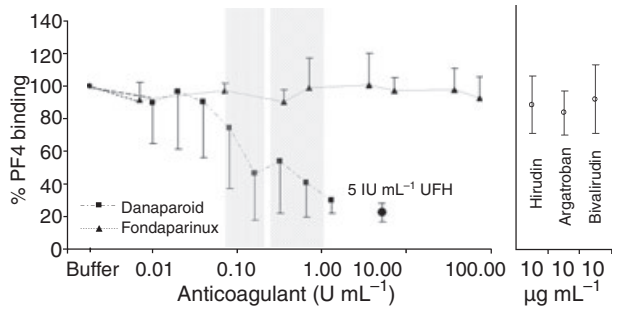

Fig. 2. Platelet factor 4 (PF4)-transfected EBNA cells were incubated with increasing concentrations of danaparoid or fondaparinux, 5 IU mL^{-1} unfractionated heparin, or 10 μg mL^{-1} hirudin, argatroban, or bivalirudin. Prophylactic-dose and therapeutic-dose concentration ranges are given as vertical and diagonal striped areas, respectively. Surface-bound PF4 without the addition of any anticoagulant was defined as 100%. Prophylactic and, especially, therapeutic-dose concentrations of danaparoid displaced PF4 from EBNA cells. Fondaparinux in prophylactic-dose and therapeutic-dose concentrations, as well as hirudin, argatroban and bivalirudin at supratherapeutic levels, had no effect. Data (mean ± SD) represent results of three experiments.

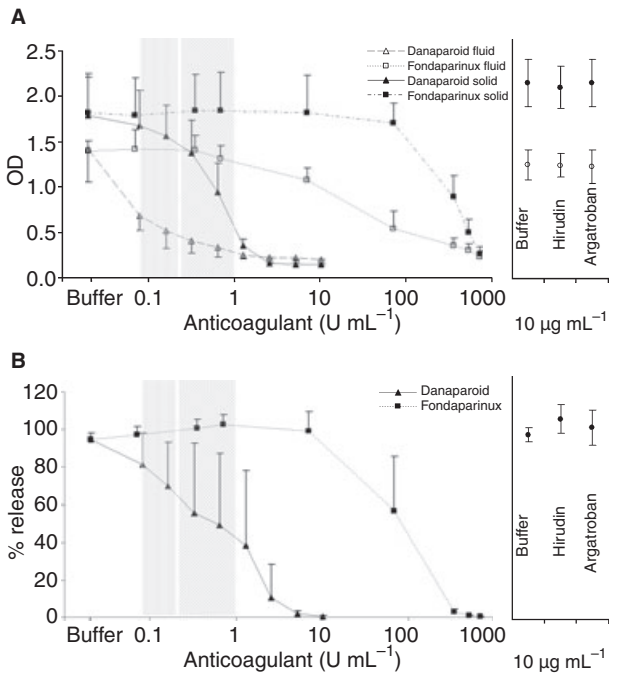

Fig. 3. Effects of different anticoagulants on the interaction of anti-PF4/heparin antibodies with PF4/heparin complexes and their capacity to activate platelets. (A) Sera from heparin-induced thrombocytopenia (HIT) patients were tested in solid-phase and fluid-phase platelet factor 4 (PF4)--heparin enzyme immunoassays (EIAs) in the presence of increasing concentrations of danaparoid or fondaparinux, or of 10 μg mL^{-1} hirudin or argatroban. Prophylactic-dose and therapeutic-dose ranges are given as vertical and diagonal striped areas, respectively. Therapeutic concentrations of danaparoid inhibited antibody binding to PF4–heparin complexes more than prophylactic concentrations in both solid-phase and fluid-phase PF4–heparin EIAs. Fondaparinux decreased optical density (OD) only at supratherapeutic concentrations. Hirudin and argatroban showed no influence. Data (mean ± SD) from five experiments are shown. (B) Sera from HIT patients were tested in a serotonin-release assay in the presence of increasing concentrations of danaparoid or fondaparinux, or of 10 μg mL^{-1} hirudin or argatroban. Prophylactic-dose and therapeutic-dose concentration ranges are given as vertical and diagonal striped areas, respectively. Therapeutic concentrations of danaparoid inhibited serotonin release more than prophylactic concentrations. Fondaparinux reduced release only at supratherapeutic concentrations. Hirudin and argatroban showed no influence. Data (mean ± SD) from five experiments are shown.

concentrations (0.64 anti-FXa U mL^{-1} = 32 μg mL^{-1}; $P = 0.001$);in the fluid-phase PF4–heparin EIA, the effect was even more pronounced (0.16 anti-FXa U mL^{-1} = 8 μg mL^{-1}, $P = 0.012$; and 0.64 anti-FXa U mL^{-1} = 32 μg mL^{-1}, $P = 0.004$) (Fig. 3A). Fondaparinux decreased the OD only at very high concentrations (~ 4-fold vs. treatment doses). Hirudin and argatroban showed no effect.

SRA

Danaparoid inhibited platelet activation by anti-PF4–heparin antibodies: at 0.16 anti-FXa U mL^{-1} (= 8 μg mL^{-1}) by 26% ($P = 0.060$), and at 0.64 anti-FXa U mL^{-1} (= 32 μg mL^{-1}) by 48% ($P = 0.046$) (Fig. 3B). Fondaparinux inhibited serotonin release only at supratherapeutic concentrations, and neither hirudin nor argatroban had any effect.

PCS

By PCS, PF4 alone showed an MHD of about 12 nm (Fig. 4). Preincubation with UFH (0.5 IU mL^{-1} = 3.3 μg mL^{-1}) increased the particle size to an MHD of 23 nm, due to PF4–heparin complex formation ($P = 0.029$) Danaparoid at therapeutic-dose concentrations (0.6 anti-FXa U mL^{-1} = 30 μg mL^{-1}; $P = 0.025$) reduced MHD, indicating disruption of PF4–heparin complexes. Fondaparinux also led to disruption of PF4–heparin complexes at supratherapeutic concentrations (> 7 anti-FXa U mL^{-1} = 10 μg mL^{-1}), whereas hirudin, argatroban and bivalirudin had no effect.

Discussion

In this study, only danaparoid at clinically meaningful (pharmacologic) concentrations inhibited activation of platelets by HIT antibodies. This effect is independent of its direct anticoagulant action, given that therapeutic concentrations of fondaparinux and the three direct thrombin inhibitors had no effect. Rather, this inhibitory effect of danaparoid [19,20] is due to its interference with the formation of the PF4-containing antigens. Danaparoid inhibits formation of PF4–heparin complexes, and binding of PF4, and of PF4–heparin complexes, to cell surfaces, and removes PF4–heparin complexes from cell surfaces, essentially by disrupting these complexes. These effects are most likely charge-related, and are especially pronounced with danaparoid, due to its low specific anticoagulant activity, which results in high concentrations of anticoagulant-inactive – but nonetheless PF4-binding – molecules for a given degree of anti-FXa activity.

We first tested whether UFH interferes with PF4 binding to platelets and how danaparoid and fondaparinux compare to

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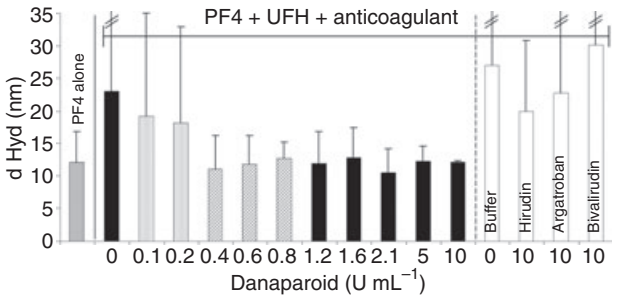

Fig. 4. Photon correlation spectroscopy of platelet factor 4 (PF4) alone and with 0.5 IU mL^{-1} unfractionated heparin (UFH) (left black bar) plus increasing concentrations of danaparoid or 10 μg mL^{-1} hirudin, argatroban, or bivalirudin. Prophylactic-dose and therapeutic-dose concentration ranges of danaparoid are given as vertical and diagonal striped bars, respectively. Danaparoid at prophylactic-dose and therapeutic-dose concentrations reduced PF4–heparin complex size. Hirudin, argatroban and bivalirudin had no significant effect. Data (mean ± SD) represent results of a minimum of three experiments per concentration of danaparoid. dHyd, hydrodynamic diameter.

UFH. Addition of PF4 resulted in binding to platelets even in the absence of any of these anticoagulants. Binding was enhanced by UFH, especially in the range of concentrations attained when prophylactic-dose UFH was given. However, higher concentrations of UFH reduced PF4 binding in a dose-dependent fashion. Thus, we have confirmed previous findings [32,33] that PF4 binding depends on UFH concentrations, following a bell-shaped curve. This is consistent with the formation of multimolecular PF4–heparin complexes at optimal molar ratios of PF4 and UFH [2,27]. Above the optimal ratio, complex formation was impaired, and eventually PF4 binding was even prevented.

The present study is in line with, and provides a potential explanation for, an interesting finding reported by Survana *et al.* [8]. In our study, maximal binding of PF4 to platelets appeared at a PF4/heparin molar ratio of 20 : 1, which is much higher than the 1 : 1 or 2 : 1 ratios that lead to complexes of maximal size in the PCS [7] and to maximal HIT antibody binding in the EIA. This, however, is the same ratio that caused the strongest anti-PF4–heparin antibody development after injection of PF4–heparin into mice [8]. Potentially, cell surface GAGs participating in complex formation on platelet membranes result in reduced amounts of the heparin that is needed for maximal PF4 binding.

When we then assessed the effects of alternative anticoagulants on PF4 binding to platelets, danaparoid enhanced PF4 binding weakly at 0.04 anti-FXa U mL^{-1} (= 2 μg mL^{-1}). This concentration is below the prophylactic-dose range (0.1–0.2 anti-FXa U mL^{-1}). However, if the drug is given in prophylactic doses, it is possible that patients attain this concentration range *in vivo*, leading to PF4–danaparoid complex formation and binding to platelets. This is in line with the empirical finding that patients with acute HIT without thrombosis benefit from therapeutic-dose rather than prophylactic-dose danaparoid [34]. Of course, this observation could also reflect differences in the dose-dependent anticoagulant effect. Moreover, the weak enhancement of PF4 binding to platelets by danaparoid could be an explanation for the frequently described *in vitro* 'cross-reactivity' of HIT antibodies to PF4–danaparoid [18]. We did not assess sera with *in vitro* cross-reactivity in this study. Thus, the underlying mechanism of *in vitro* cross-reactivity remains unclear. Fondaparinux did not increase PF4 binding to platelets, although at very high, clinically irrelevant concentrations (> 7 anti-FXa U mL^{-1}), it inhibited PF4 binding to platelets.

In acute HIT, heparin has already formed antigenic complexes on platelet surfaces. We therefore next addressed the question of whether alternative anticoagulants affect PF4–heparin complexes already bound to platelets. As expected, heparin at high (supratherapeutic) concentrations displaced PF4–heparin complexes from platelets. Also, danaparoid was highly efficient at removing PF4–heparin complexes from the platelet surface, showing near-maximal effects in the upper range of therapeutic-dose concentrations. The mechanism is most likely disruption of PF4–heparin complexes by high concentrations of danaparoid. According to PCS, danaparoid did not form measurable complexes with PF4 (not shown), but reduced PF4–heparin complex size in a dose-dependent fashion, being most effective at higher (therapeutic) levels. This provides an explanation for the finding that danaparoid inhibits platelet aggregation and thromboxane B_2 production induced by HIT antibodies [19,20]. In contrast, neither fondaparinux (prophylactic dose or therapeutic dose) nor the three direct thrombin inhibitors (hirudin, argatroban, and bivalirudin) had any effect on binding of preformed PF4–heparin complexes to platelet surfaces. As HIT also affects endothelial cells [35] and monocytes [36], we next assessed whether the different anticoagulants also affect binding of PF4 to cell types other than platelets. For this, we used a genetically modified, PF4-expressing cell line, coexpressing the PF4 receptor CXCR3-B [37], which is a model used to study the effects of PF4 binding to its receptor on nucleated cells. With this cell line, we obtained similar results as per the platelet experiments. This clearly demonstrates that the effects of danaparoid on PF4 binding are not restricted to platelets.

After demonstrating that different alternative anticoagulants had different effects on PF4–platelet interactions, as well as on the disruption of preformed PF4–heparin complexes, we used the experimental systems of purified PF4–heparin complexes in solid-phase and in fluid-phase EIAs, and the platelet SRA, to assess whether the anticoagulants also have different effects on the interaction of HIT antibodies with PF4–heparin complexes, as well as their capacity to activate platelets. Consistent with the PF4 binding studies and the PCS studies, binding of HIT antibodies to PF4–heparin complexes was reduced in both solid-phase and fluid-phase PF4–heparin EIAs in the presence of prophylactic and therapeutic concentrations of danaparoid, whereas the other alternative anticoagulants had no effect. Even more importantly, danaparoid at prophylactic and therapeutic concentrations also inhibited serotonin release induced by HIT antibodies, whereas fondaparinux only had an effect at supratherapeutic concentrations. In accordance with

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prior studies [7], the direct thrombin inhibitors showed no influence.

A weakness of our study is its pure *in vitro* design, including the use of plasma-free systems. *In vivo*, heparin has a low bioavailability ($\sim$ 20%) for its anticoagulant effects, because of non-specific protein binding. Thus, it is likely that much higher concentrations of heparin are needed for PF4 complex displacement *in vivo* than in a purified system. As UFH was most efficacious in displacing PF4 at a concentration of $\sim$ 1 IU mL^{-1}, this corresponds to an *in vivo* concentration of approximately 5 IU mL^{-1} (i.e. above its usual therapeutic range). In contrast, danaparoid has a high bioavailability ($\sim$ 100%), and thus PF4 binding of danaparoid *in vitro* probably reflects the *in vivo* situation.

In conclusion, our study supports the concept that danaparoid is not only an anticoagulant that decreases thrombin generation in HIT but – in contrast to all other alternative anticoagulants – one that also inhibits HIT pathogenesis at clinically relevant drug concentrations, by interfering with PF4–heparin complex formation and PF4 binding to cells in a charge-dependent manner. The study provides an additional pathophysiologic rationale that underscores the clinical recommendation [10] to treat patients with acute HIT with therapeutic-dose danaparoid even in situations of isolated thrombocytopenia.

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Disclosure of Conflict of Interests

T.E. Warkentin is a consultant for, and has received lecture honoraria and research funding from, GlaxoSmithKline and Organon International (part of Schering-Plough), A. Greinacher is a consultant for Organon Int and Mitsubishi Pharma and received speakers honoraria from Organon Int., GlaxoSmithKline and Pharmion.

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6.3 Platelet factor 4 binds to bacteria, inducing antibodies cross-reacting with the major antigen in heparin-induced thrombocytopenia

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Platelet factor 4 binds to bacteria, inducing antibodies cross-reacting with the major antigen in heparin-induced thrombocytopenia

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A clinically important adverse drug reaction, heparin-induced thrombocytopenia (HIT), is induced by antibodies specific for complexes of the chemokine platelet factor 4 (PF4) and the polyanion heparin. Even heparin-naive patients can generate anti-PF4/heparin IgG as early as day 4 of heparin treatment, suggesting preimmunization by antigens mimicking PF4/heparin complexes. These antibodies probably result from bacterial infections, as (1) PF4 bound charge-dependently to various bacteria, (2) human heparin-induced anti-PF4/heparin antibodies cross-reacted with PF4-coated *Staphylococcus aureus* and *Escherichia coli*, and (3) mice developed anti-PF4/heparin antibodies during polymicrobial sepsis without heparin application. Thus, after binding to bacteria, the endogenous protein PF4 induces antibodies with specificity for PF4/polyanion complexes. These can target a large variety of PF4-coated bacteria and enhance bacterial phagocytosis in vitro. The same antigenic epitopes are expressed when pharmacologic heparin binds to platelets augmenting formation of PF4 complexes. Boosting of preformed B cells by PF4/heparin complexes could explain the early occurrence of IgG antibodies in HIT. We also found a continuous, rather than dichotomous, distribution of anti-PF4/heparin IgM and IgG serum concentrations in a cross-sectional population study (n = 4029), indicating frequent preimmunization to modified PF4. PF4 may have a role in bacterial defense, and HIT is probably a misdirected antibacterial host defense mechanism. (*Blood*. 2011;117(4): 1370-1378)

Introduction

The chemokine platelet factor 4 (PF4, CXCL4) is stored within platelet α-granules[1] and released during platelet activation. Although its biologic role is poorly understood, PF4 commands attention in clinical medicine because it binds charge-dependently to the anticoagulant heparin, one of the most frequently used anticoagulants in clinical medicine, thereby neutralizing heparin's anticoagulant effect,[2,3] but also forming highly antigenic multimolecular complexes.[4-6] The resulting antibody response[7] induces the most frequent immune-mediated adverse drug reaction involving human blood cells, heparin-induced thrombocytopenia (HIT).[8] The pathogenic antibodies bind to PF4/heparin complexes at the platelet surface, and the resulting immune complexes induce Fc-receptor–mediated platelet activation and enhanced thrombin generation.[8] In a subset of patients, this causes thrombocytopenia and triggers paradoxical thrombosis, which is aggravated by continuation of heparin treatment.

The immune response of HIT has several atypical features. Even in patients who receive heparin for the first time, there is rapid induction (as early as 4 days) of anti-PF4/heparin antibodies of IgG isotype. Moreover, IgG antibodies are the predominant class of immunoglobulins formed.[9,10] We and others discovered that up to 50% of patients after cardiopulmonary bypass surgery and 20% to 30% of orthopedic surgery patients (many of whom have not been previously exposed to heparin) developed anti-PF4/heparin IgG antibodies in the second week after heparin exposure.[11-13]

These findings are unusual for a primary immune response, in which predominant formation of IgM, followed by later and weaker response of IgG, would be expected. In addition, serum from apparently healthy persons can contain anti-PF4/heparin antibodies, even without previous exposure to pharmacologic heparin.[14,15] Recently "spontaneous" HIT has been described, in which patients develop an illness clinically and serologically mimicking HIT with strongly reacting anti-PF4/heparin IgG antibodies in the absence of any exposure to heparin.[16-18] All these clinical observations suggest exposure to the antigen induced by a binding partner of PF4 different from pharmacologic heparin.

It is known that anti-PF4/heparin antibodies are not heparin-specific, as they also recognize PF4 bound to certain other polyanions in vitro.[19-21] Anti-PF4/heparin antibodies can also be induced in vivo by certain nonheparin polyanions, such as hypersulfated chondroitin sulfate.[22] These observations raise the possibility that in HIT the immune system might have encountered PF4 complexes earlier, perhaps because of binding of PF4 to naturally occurring nonheparin polyanions. It was therefore an intriguing observation that 3 of the 5 reported patients with the spontaneous HIT syndrome had histories of recent bacterial infection. The cell

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surface of bacteria could be a potential natural source for PF4-binding polyanions.[23,24] In this study, we assessed whether PF4 can bind to bacteria, thereby exposing the epitope recognized by antibodies induced in patients during heparin treatment, and whether bacteria can induce anti-PF4/heparin antibodies in a mouse model.

Methods

Bacterial strains

Escherichia coli JM109 (Promega), *Neisseria meningitidis* MC58Δ*siaD*,[25] *Staphylococcus aureus* SA113*spa* (protein A–deficient strain, provided by Dr Goerke, Universität Tübingen, Tübingen, Germany), *Streptococcus pneumoniae* NCTC 10319 (serotype 35A),[26] and *Listeria monocytogenes* L028 Sr 1/2c[27] were grown in Todd-Hewitt broth (0.5% yeast extract; Roth) until mid-log phase.[28] Fluorescein isothiocyanate (FITC)–labeled *E coli* (K-12 strain) were obtained from Invitrogen.

PF4 biotinylation

We biotinylated human (hu)-PF4 from platelets and recombinant murine (mu)-PF4 (Chromatec GmbH) as described.[29] Briefly, Heparin Sepharose (GE Healthcare) was washed 3 times with phosphate-buffered saline (PBS), pH 7.4, mixed with PF4 for 15 minutes, and then left overnight at 4°C. The PF4-heparin-Sepharose suspension was mixed and incubated with biotin-XX (1 hour at room temperature; Invitrogen), washed (0.8M NaCl, 20mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 5mM ethylenediaminetetraacetic acid, pH 7.4), and biotinylated PF4 eluted with high salt buffer (2M NaCl, 20mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 5mM ethylenediaminetetraacetic acid, pH 7.4). The concentration of biotinylated PF4 was determined by a bicinchoninic acid protein assay kit using bovine serum albumin as standard (Sigma-Aldrich).

PF4 binding to bacteria

We incubated *E coli*, *N meningitidis*, *S aureus*, *S pneumoniae,* and *L monocytogenes* (30 minutes; 4°C) with biotinylated hu-PF4 (0, 1.25, 2.5, 5, 10, 20, and 40 μg/mL) or mu-PF4 (*E coli, S aureus*), washed the bacteria (PBS + 0.05% bovine albumin; 3000*g*, 5 minutes, 4°C), incubated the bacteria (30 minutes; 4°C) with peridinin chlorophyll protein-Cy5.5 conjugated streptavidin (BD Biosciences), washed them again, and fixed them (1% paraformaldehyde; 20 minutes, 4°C). We analyzed PF4 binding by flow cytometry (Cytomics FC 500, Beckman Coulter). The geometric mean fluorescence intensity multiplied by the percentage of labeled bacteria constituted binding activity. As a control, we also assessed binding of nonbiotinylated PF4 to bacteria, which was visualized with an FITC-labeled polyclonal rabbit anti–hu-PF4 antibody.[30] Results did not differ from the ones obtained with biotinylated PF4.

In addition, we tested biotinylated hu-PF4 binding (20 μg/mL) to *S aureus* SA113*spa* in the presence of increasing concentrations (0, 12.5, 25, 50, and 100 μg/mL) of heparin (150 IU/mg; Braun) or dextran sulfate (Sigma-Aldrich).

PF4/heparin antibody assays

We performed the hu-PF4/heparin-enzyme immunoassay (EIA) with human sera of healthy volunteers, of patients who were immunized during heparin treatment and with eluates and adsorbates of PF4-coated or uncoated bacteria as described.[31]

In addition, we analyzed mouse sera using recombinant mu-PF4/heparin complexes as antigen and the respective mouse antibody specific conjugates: goat anti–mouse IgG Fc-specific peroxidase (Sigma-Aldrich) or goat anti–mouse IgM μ-chain–specific peroxidase (Dianova). All sera were tested for inhibition by soluble excess unfractionated heparin (100 IU/mL), which disrupts PF4/heparin complexes and for binding to PF4 alone.

The heparin-induced platelet activation (HIPA) test was performed as described.[32]

Antibody purification (adsorption and elution experiments)

We preincubated *S aureus* SA113*spa* or *E coli* JM109 with hu-PF4 (20 μg/5 × 10^7 bacteria, 30 minutes, 4°C) or buffer (PBS, pH 7.4), washed (3000*g*, 5 minutes, 4°C) and incubated (30 minutes, 4°C) them with diluted human serum of patients known to contain anti-PF4/heparin IgG antibodies (n = 5 per strain), and washed them again to remove unbound antibodies. We eluted bound antibodies with glycine buffer (0.1M, pH 2.7; 5 minutes at room temperature), centrifuged the bacteria, and neutralized the supernatant (eluate) with Tris buffer (1M, pH 9). We tested untreated sera and eluates by hu-PF4/heparin IgG EIA and HIPA test. To exclude nonspecific antibody binding, we also processed human sera containing anti–human leukocyte antigens–class I antibodies (n = 1), anti–human platelet antigen-1a alloantibodies (n = 2), and an anti-glycoprotein Ib/IX platelet autoantibody (n = 1) identically and measured IgG binding of the eluates to human platelets expressing the respective antigens assessed by flow cytometry and by a glycoprotein-specific EIA, the monoclonal antibody immobilization of platelet antigens method monoclonal antibody–specific immobilization of platelet antigens.[33]

Phagocytosis assay

This assay was modified from the phagotest kit (Orpegen Pharma). We diluted heat-inactivated (56°C, 45 minutes) human sera (1:50) of patients containing anti-PF4/heparin IgG antibodies or control sera and preadsorbed them with *E coli*–FITC (non–PF4-coated; 15 minutes, 37°C; 4 times), incubated the preadsorbed sera (n = 4) with hu-PF4–coated or –noncoated *E coli*–FITC (16 hours, 4°C), and then incubated (10 minutes, 37°C) the bacteria with whole blood cells, which we obtained by washing hirudinized blood of healthy volunteers twice (PBS; 300*g*, 5 minutes, 4°C) and then incubated on ice for 10 minutes. We quenched fluorescent signals from *E coli*–FITC attached to the cell surface with trypan blue (5 minutes, ice bath; Sigma-Aldrich). After this incubation step, we washed the cells twice and lysed the erythrocytes with FACS lysing solution (7 minutes at room temperature; BD Biosciences), washed the remaining cells with PBS (300*g*, 5 minutes, 4°C), stained their DNA with propidium iodide (10 minutes, ice bath; Fluka BioChemica), and analyzed the cells by flow cytometry. We recorded the mean fluorescence intensity (MFI) of FITC-positive polymorphonuclear leukocytes (PMNs) as a measure for bacterial phagocytosis.

Polymicrobial sepsis model

We implanted an 18-G stent (Ohmeda AB) into the ascending colon of ketamine-anesthetized female C57BL/6 mice (age 7 weeks; Charles River), which induced colon ascendens stent peritonitis (CASP) by allowing controlled leakage of feces into the abdominal cavity.[34,35] At day 1, 3, 7, and 14 after CASP, we drew blood from the retroorbital sinus. In sham operations, we fixed the stent outside the ascending colon and took blood at days 1, 7, and 14.

SHIP

Study of Health in Pomerania (SHIP) is a cross-sectional population-based survey conducted in northeast Germany.[36] Details of the population assessed in this study are given elsewhere.[37] Of 4310 participants, we assessed the sera of 4029 persons of whom serum was available by hu-PF4/heparin-EIA. Heparin administration or any in-hospital treatment within the last 12 months before blood drawing was excluded for all participants. This time period is sufficient to exclude antibody persistence induced by earlier application of pharmacologic heparin, as anti-PF4/heparin antibodies usually disappear within 3 to 6 months after the immune response.[38]

Statistical analysis

We calculated differences between the eluates of PF4 and buffer-preincubated bacteria in the adsorption/elution experiments by paired *t* test. For the phagocytosis assay, we compared samples by paired *t* test. We analyzed antibody production in the mouse model using Wilcoxon rank-sum test. All samples tested in PF4/heparin-EIA were compared with

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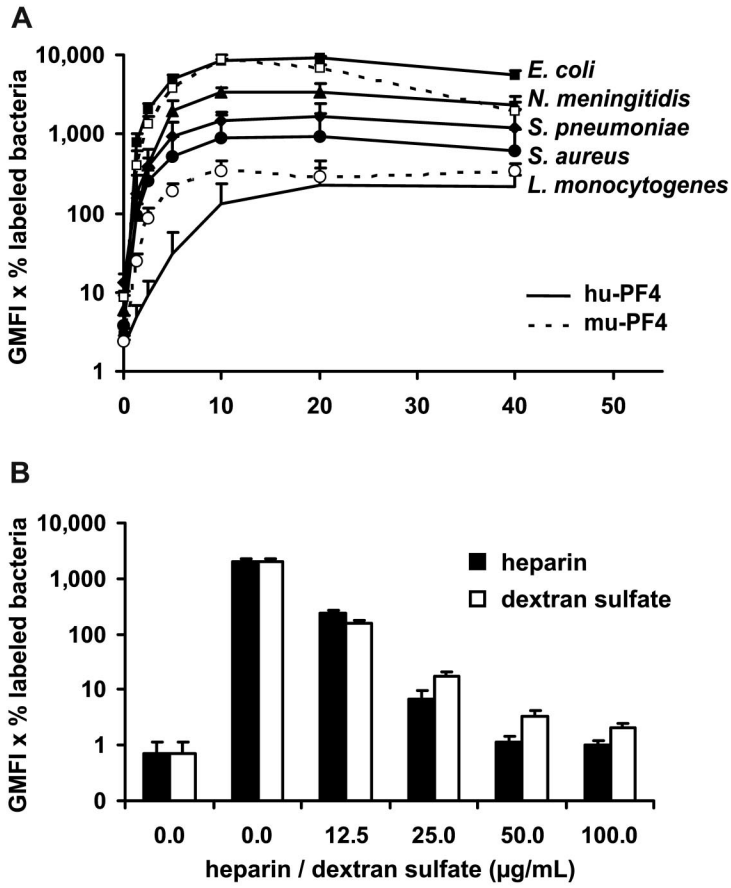

Figure 1. PF4 binds to Gram-positive (*S aureus, S pneumoniae,* and *L monocytogenes*) and Gram-negative bacteria (*E coli* and *N meningitidis*). (A) PF4 binding to Gram-positive and Gram-negative bacteria is dose-dependent and saturates at 20 μg/mL. Binding capacity for PF4 differs between bacterial strains, with *E coli* showing the highest binding capacity. Each bacterial strain was incubated with increasing concentrations of biotinylated hu-PF4 (solid symbols, all bacteria) or mu-PF4 (open symbols, *E coli* and *S aureus*). PF4 binding was detected with peridinin chlorophyll protein–Cy5.5-labeled streptavidin using flow cytometry and expressed as geometric mean fluorescence intensity (GMFI) multiplied by the percentage of labeled bacteria. Data are mean ± SD of 3 independent experiments. (B) PF4 binding to *S aureus* SA113*spa* is inhibited in a dose-dependent manner by heparin and dextran sulfate demonstrating charge dependency. *S aureus* SA113*spa* was incubated with buffer or with 20 μg/mL biotinylated hu-PF4 alone or in the presence of increasing concentrations of heparin or dextran sulfate (0, 12.5, 25, 50, and 100 μg/mL). PF4 binding was detected with peridinin chlorophyll protein–Cy5.5-labeled streptavidin using flow cytometry and expressed as GMFI multiplied by the percentage of labeled bacteria. Data are mean ± SD of 3 independent experiments.

their reaction in the presence of excess heparin and in binding to PF4 alone using paired *t* test. *P* less than .05 was considered statistically significant.

Ethics

All volunteers gave written informed consent in accordance with the Declaration of Helsinki. All experiments were performed according to the German animal safety regulations and approved by the local animal protection authority.

Results

PF4 binding to bacteria

As bacteria display negatively charged molecules on their surfaces, we assumed that these molecules could be binding partners for PF4. We found that hu-PF4 bound in a dose-dependent manner to Gram-positive bacteria, such as *S aureus*, *S pneumoniae*, and *L monocytogenes*, and Gram-negative bacteria, such as *E coli* and *N meningitidis.* Saturation of binding was reached at 20 μg/mL (n = 3 each) of PF4 (Figure 1A), with *E coli* showing the highest binding capacity. Similarly to hu-PF4, mu-PF4 bound to *S aureus* and *E coli* (Figure 1A). Binding of hu-PF4 to *S aureus* was inhibited by heparin (n = 3) and dextran sulfate (n = 3), suggesting a charge-dependent interaction between PF4 and the bacteria (Figure 1B).

Binding of anti-PF4/heparin antibodies to PF4-coated bacteria

To assess whether human anti-PF4/heparin antibodies bind to PF4/polyanion complexes on the bacterial surface, we used hu-PF4–coated bacteria to affinity purify (by adsorption and elution) IgG antibodies of sera (n = 5) from patients with HIT. These patients were known to contain anti-PF4/heparin IgG antibodies. After 3 or 4 adsorption steps, anti-PF4/heparin antibodies were depleted from the human sera (Figure 2A-B insets). The IgG antibodies eluted from hu-PF4-coated bacteria reacted with hu-PF4/heparin complexes in the PF4/heparin-EIA (Figure 2). All controls showed the expected results (Figure 2): (1) the affinity-purified antibodies did not react with hu-PF4 alone; (2) excess heparin (100 IU/mL), which disrupts PF4/heparin complexes, inhibited anti-PF4/heparin antibody binding; (3) non–PF4-coated bacteria did not bind the anti-PF4/heparin antibodies; and (4) platelet-reactive antibodies with other specificities (antihuman leukocyte antigens-class I antibodies, n = 1; anti–human platelet antigen-1a alloantibodies, n = 2; and an anti–glycoprotein Ib/IX platelet autoantibody, n = 1) were not affinity-purified by PF4-coated bacteria. The eluates of PF4-coated bacteria incubated with these antiplatelet antibodies showed negligible IgG binding to platelets as analyzed by flow cytometry (1.6%–4.2% binding of the original serum) and all optical densities (ODs) were below the cutoff of 0.2 in the glycoprotein specific EIA.

The affinity-purified anti-PF4/heparin antibodies also activated platelets in a functional test: HIPA assay, at low (0.2 IU/mL) but not at high heparin concentrations (100 IU/mL; 5 of 5 eluates of hu-PF4-coated *E coli* and 3 of 5 eluates of hu-PF4-coated *S aureus*). Probably the concentration of the antibodies in 2 of 5 eluates of PF4-coated *S aureus* was not high enough for providing a solid functional response in the HIPA test. Because *S aureus* had a lower PF4 binding capacity than *E coli*, it is possible that also less anti-PF4/heparin antibodies bound.

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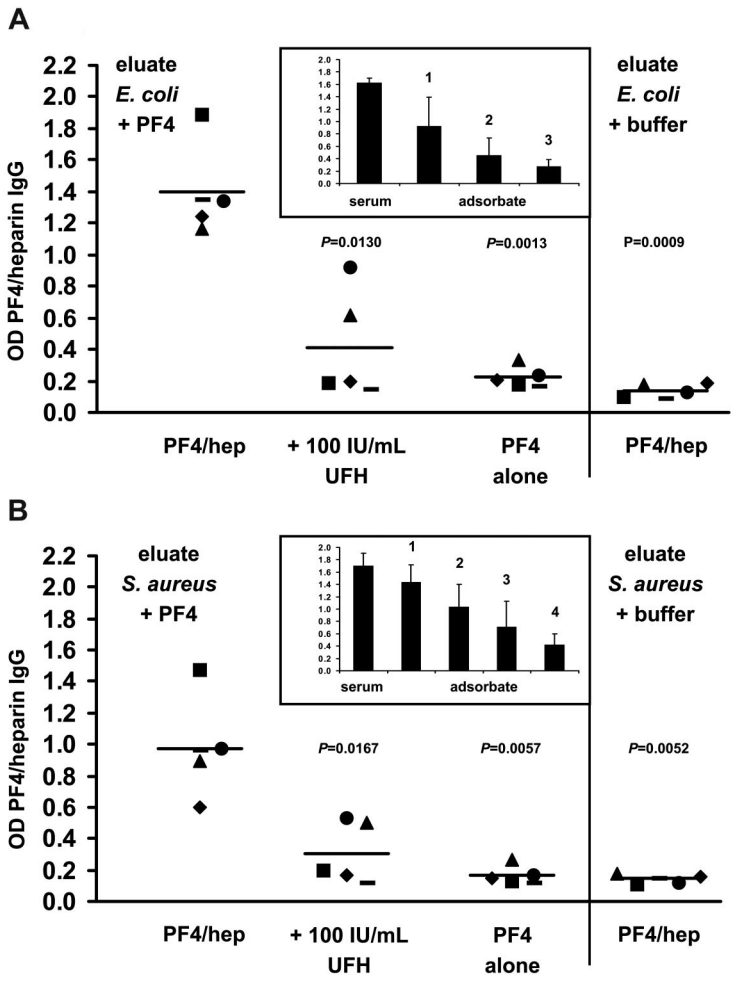

Figure 2. Anti-PF4/heparin IgG from sera of patients with HIT binds to PF4-coated bacteria but not to bacteria alone. From sera of 5 patients with HIT, known to contain anti-PF4/heparin antibodies, IgG antibodies were affinity purified using hu-PF4-precoated bacteria. (Insets) Depletion of the anti-PF4/heparin antibodies from the original serum (n = 5) by sequential incubation with hu-PF4–coated bacteria (mean ± SD). In the main panels, each symbol represents the reactivity of the affinity-purified antibodies of one serum; mean values are given as horizontal lines. Reactivities of antibodies affinity purified by hu-PF4-coated *E coli* JM 109 (A) or by hu-PF4-coated *S aureus* SA113*spa* (B). Reactivity of the purified antibodies with hu-PF4/heparin complexes (first column) was inhibited by excess heparin (100 IU/mL unfractionated heparin [UFH], column 2), which disrupts PF4/heparin complexes. Accordingly, antibodies did also not react with hu-PF4 alone (column 3). Non–PF4-coated bacteria served as control for unspecific binding of the antibodies to bacteria alone (column 4).

A mouse model for anti-PF4/heparin antibody induction by bacterial infection

To test whether bacteria can induce anti-PF4/heparin antibodies in vivo, we used a mouse model of polymicrobial sepsis. Sublethal polymicrobial bacterial peritonitis was induced by insertion of a stent into the ascending colon of mice (CASP). Non–heparin-treated C57BL/6 mice developed anti–mu-PF4/heparin IgM starting at day 3 (OD: mean ± SD, 0.738 ± 0.191 vs 0.234 ± 0.117 at day 1, $P = .0051$; Figure 3A). IgM concentrations increased at day 7 (OD: mean ± SD, 1.389 ± 0.457, $P = .0051$ vs day 1); subsequently, titers decreased somewhat to day 14 but remained elevated throughout the observation period (OD: mean ± SD, 0.914 ± 0.215, $P = .0081$ vs day 1). Antibody binding was significantly lower in the presence of excess heparin and also when mu-PF4 alone was coated. Sham-operated mice also showed a slight increase in OD levels, which, however, were significantly lower compared with those of CASP mice (day 7, $P = .0082$; day 14, $P = .0003$), whereas the untreated control mice showed negligible anti–mu-PF4/heparin IgM production.

IgG binding to mu-PF4/heparin increased at day 14 in sera of CASP mice (OD: mean ± SD, 0.543 ± 0.287, $P = .0024$ vs day 1; Figure 3B) and was significantly higher than in sera of sham-operated mice ($P = .0149$). Binding was reduced by excess heparin ($P = .0280$). The untreated control group showed no anti–mu-PF4/heparin IgG production. This pattern is compatible with a primary immune response, with early IgM and a delayed weak IgG response.

The IgG antibodies also bound to mu-PF4 alone, a feature seen in some patients with HIT.[39]

Biologic effects of anti-PF4/heparin antibodies

To address the question of whether anti-PF4/heparin antibodies have biologic effects on the clearance of bacteria, we incubated hu-PF4–coated bacteria with hu-PMNs. Anti-PF4/heparin-IgG containing human serum significantly enhanced phagocytosis of PF4-coated *E coli* by PMNs (n = 4, MFI, 1151.3 ± 207.8) compared with noncoated *E coli* (n = 4; MFI, 286.7 ± 34.9, $P = .0052$) (Figure 4). Control serum did not increase phagocytosis of hu-PF4-coated *E coli* (n = 4; 453.1 ± 122.3) compared with noncoated *E coli* (n = 4; 259.5 ± 7.4, $P = .0541$). Phagocytosis of hu-PF4-coated *E coli* was significantly higher after preincubation with anti-PF4/heparin-IgG-containing human serum than with control serum, which did not contain anti-PF4/heparin antibodies ($P = .0012$). hu-PF4 alone, without serum, had no effect on phagocytic activity (MFI < 200).

Prevalence of anti-PF4/heparin antibodies in the general population

To provide further evidence that anti-PF4/heparin antibodies can be induced by means other than heparin treatment (eg, PF4/bacteria

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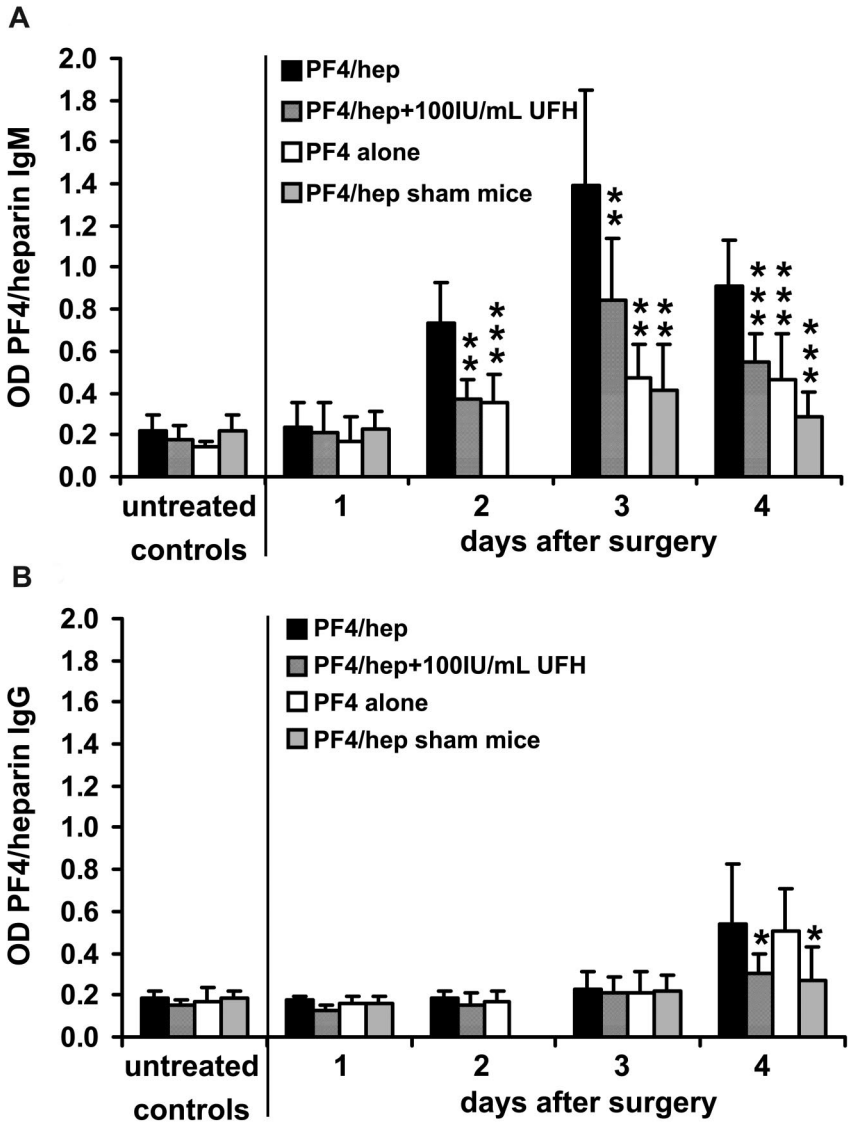

Figure 3. Mice with polymicrobial sepsis develop anti-PF4/heparin antibodies. C57BL/6 mice underwent CASP, which caused polymicrobial sepsis, or were sham-operated. At 1, 3, 7, and 14 days after surgery, sera were tested for anti–mu-PF4/heparin IgM (A) and IgG (B) as described in "P4/heparin antibody assays." Mice with polymicrobial sepsis (black bars) developed anti–mu-PF4/heparin IgM from day 3 and anti–mu-PF4/heparin IgG from day 14. Binding of IgM and IgG was reduced by excess heparin (100 IU/mL, striped bars). When mu-PF4 alone was coated (open bars), IgM antibodies showed minimal binding, but the IgG antibodies also reacted. Antibody titers of mice with CASP were always higher than those of sham-treated mice (gray bars). At each time point, at least 6 mice were assessed (days 1, 3, and 7: n = 6 each for CASP and sham-operated mice; day 14: n = 8 for CASP, n = 11 for sham). Data represent mean OD ± SD. All comparisons of OD values were performed between the OD values obtained at the same experimental day using as reference the values obtained when the sera of CASP mice where incubated with PF4/heparin complexes (black bars). *$P < .05$; **$P < .01$; ***$P < .001$.

complexes), we systematically assessed the presence of anti-PF4/heparin antibodies in the general population with a very low likelihood of recent heparin treatment. We tested serum samples obtained in a cross-sectional population study[37] (n = 4029) using a hu-PF4/heparin-EIA. The normal population showed a continuous, rather than a dichotomous, distribution of reactivities (Figure 5) with no significant effect of age ($\beta = -0.002$, $P = .128$) or sex ($\beta = 0.031$, $P = .472$). In total, 6.1% of the normal population showed an OD higher than 0.5 for IgG (Figure 5A) and 18.8% for IgM (Figure 5B). In 72% of sera with OD higher than 0.5 for IgG (total 4.4%) and in 42% of sera with OD higher than 0.5 for IgM (total 7.9%), the antibody binding to hu-PF4/heparin was inhibited by a high concentration of heparin (100 IU/mL) by more than 30%. Inhibition by high heparin indicates specificity for hu-PF4/heparin complexes because an excess of heparin disrupts the hu-PF4/heparin complexes.[4,40,41]

Discussion

In this study, we have shown that PF4 binds to bacteria and forms antigenic complexes on bacterial surfaces. These complexes are recognized by anti-PF4/heparin antibodies from heparin-treated patients, who developed the adverse drug effect HIT. The epitopes on bacterial-bound PF4 are thus similar or identical to those formed by PF4/heparin complexes on administration of pharmacologic heparin. These findings may therefore explain the unusual time course of the immune reaction underlying one of the most frequent and dangerous antibody-mediated adverse drug reactions, HIT, with occurrence of PF4/heparin IgG antibodies as early as day 5 after start of heparin treatment.[9,10,38] We propose that antibodies induced in a primary immune response (eg, against PF4-coated

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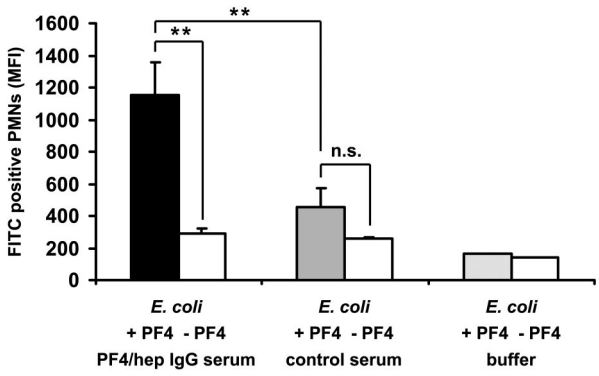

Figure 4. PF4 and anti-PF4/heparin antibodies enhance bacterial phagocytosis. FITC-labeled *E coli* were preincubated with hu-PF4 (black and gray bars) or with buffer (open bars) and additionally with heat-inactivated human serum, which did (black bar) or did not (gray bar) contain anti-PF4/heparin IgG (preadsorbed with *E coli* alone), or with buffer alone (open bar). After preincubation, the bacteria were subjected to a whole blood phagocytosis assay using flow cytometry. The figure shows the MFI of FITC-positive PMNs, which is a marker for phagocytosed *E coli*. Data are mean ± SD of 4 different sera representative of 2 independent experiments. $^{**}P < .01$. n.s. indicates not significant.

invasive bacteria) cross-react with PF4/heparin complexes formed on the platelet surface during heparin treatment. As a consequence, treatment with this anticoagulant boosts the original antibody response and causes a secondary immune reaction to PF4/heparin-coated platelets.

It is well known that the epitopes recognized by anti-PF4/heparin antibodies are formed on multimolecular, linear complexes of PF4 and heparin.[4,6] These antigenic epitopes on PF4 are exposed when the positive charge of PF4 is neutralized by polyanions, such as heparin, leading to linear, ridge-like complexes, in which the distance between single PF4 tetramers is narrowed to 3 to 5 nm.[5,42] Here we show that PF4 binds to various nonpathogenic and pathogenic Gram-positive and -negative bacteria, including *S aureus* and *E coli* (Figure 1). This interaction is charge-dependent because both dextran sulfate and heparin are able to displace PF4 from the bacterial surface. Strikingly, PF4 attached to bacterial cell surfaces exposes epitopes that bind anti-PF4/heparin antibodies from sera of patients who developed these antibodies during heparin treatment (Figure 2). The antibodies can even be "affinity purified" from patient sera using PF4-coated bacteria, which indicates that the same PF4 epitopes are formed on the bacterial surface.

In a standard mouse model of polymicrobial bacterial sepsis (CASP),[34] which permits assessment of the immune response over several weeks, we tested whether bacteria could induce the immune response to PF4/polyanions. Although mice did not receive any heparin before or during the experiment, they generated anti-PF4/heparin–reactive IgM antibodies within 3 days and low titers of anti-PF4/heparin IgG within 14 days after induction of bacterial infection (Figure 3). This reflects the typical features of a primary immune response in 7-week-old mice, which probably did not experience major bacterial infection before the experiment.

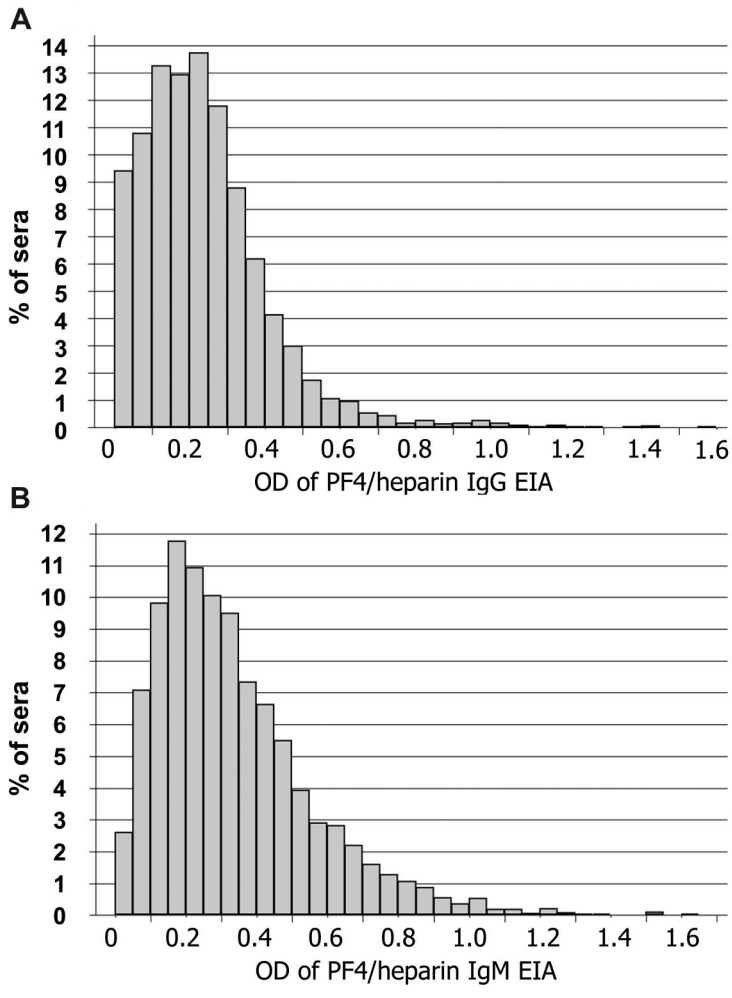

Figure 5. Anti-PF4/heparin antibodies are present in the normal population. Sera of participants (n = 4029) from the SHIP were tested for IgM- and IgG-binding to hu-PF4/heparin complexes using EIA as described in "P4/heparin antibody assays." The results for anti-PF4/heparin IgG (A) and IgM (B) antibodies are shown. The distribution of antibody reactivities as measured by OD values represented a semilogarithmic continuum rather than 2 separate populations of immunized and nonimmunized persons. This is consistent with the concept that the general population is frequently exposed to PF4/heparin-like antigen complexes, which results in a continuum of the immune response. The OD values are arbitrary units. For comparison: with the same assay, strong reacting anti-PF4/heparin antibodies from patients with HIT reacted in an OD range of 1.0 to 2.0.

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The conducted experiments do not rule out any other cause of preimmunization against PF4/polyanion complexes in humans. For instance, human cell surfaces also expose negatively charged polyanions, such as heparan sulfate and chondroitin sulfate. However, these polyanions have a much lower charge density than pharmacologic heparin.[43] Although PF4 binds to these human cell surface glycosaminoglycans, their lower charge density does not neutralize the strong positive charge of PF4 to an extent that would allow formation of the antigenic clusters recognized by anti-PF4/heparin antibodies. Indeed, heparan sulfate even disrupts the PF4/heparin complexes,[30] and heparan sulfate is the main constituent of an alternative anticoagulant (danaparoid) approved for further anticoagulation in patients with HIT.[44] Other than endothelial cell heparan sulfate, heparin stored in mast cells is more negatively charged. However, the amount of mast cell heparin released into the circulation is minimal under physiologic conditions. Indeed, if released in higher doses, it is inducing anaphylactoid reactions. Thus, the concentrations of mast cell-derived heparin in the circulation reached under physiologic conditions are probably not high enough to provide the necessary molar ratio for formation of the PF4/heparin complexes. Several groups have found these complexes to occur in vitro at a molar ratio of heparin to PF4 of 1:1 to 1:4.[5,6]

The anti-PF4/heparin antibodies may even have a biologic function as they significantly enhanced phagocytosis of PF4-coated bacteria compared with uncoated bacteria (Figure 4). Thus, the immune response to PF4/polyanion complexes could be an example for a host defense mechanism. PF4 mediates this response by acting as a bacteria-targeting marker protein. The PF4-coated bacteria are able to induce a humoral immune response against PF4 complexes, which compose a relatively narrow range of specificities. The resulting antibodies, however, can bind to a wide variety of bacterial species, even when not previously encountered by the host immune system. This is achieved through the ability of the highly cationic PF4 molecules to bind to the anionic cell walls of numerous bacterial species. It is conceivable that this anti-PF4/heparin immune response is an ancient host defense mechanism at the interface between innate and adaptive immunity. Once these anti-PF4/polyanion antibodies are formed, they can react with other bacteria even if not previously encountered by the host immune system. To our knowledge, this is the first example of a mechanism based on "labeling" pathogens with an endogenous human protein by forming linear complexes after binding to bacteria, thereby triggering a specific humoral immune response. The immune response in HIT has further interesting features, which would be consistent with an evolutionary old type of immune response. There is no clear restriction to IgG, but IgM and IgA antibodies are generated at the same time.[9] Furthermore, the antibody titers rapidly decrease within several weeks.[9,38] This might also explain the lack of memory B cells for anti-PF4/heparin IgG in patients who developed anti-PF4/heparin antibodies after cardiac surgery.[45] A potential B-cell population that might show these features are marginal zone B cells. However, this requires further studies, and our mouse model might be instrumental for identifying the involved B-cell population as it is the only model with "natural" formation of anti-PF4/heparin antibodies.

Further indirect evidence for the involvement of endogenous PF4 in sepsis has also recently been shown by enhancing survival after LPS challenge depending on activated protein C generation.[46]

However, the biologic function of the PF4/polyanion immune response induced by bacteria was not the primary focus of our study, and future studies have to address the biologic role of these antibodies in bacterial sepsis. Because mechanisms of bacterial defense are highly redundant, the absolute contribution of anti-PF4/heparin antibodies to survival is probably relatively low.

Preimmunization by PF4-coated bacteria could also explain previous observations of natural anti-PF4/heparin antibodies in non–heparin-treated persons.[14,15] The systematic assessment of natural anti-PF4/heparin antibodies in a large cross-sectional population study, the SHIP study,[37] indicated the presence of these antibodies in the normal population in a continuum of reactivities from negative to positive, without a dichotomous pattern (Figure 5). This suggests that a considerable proportion of the normal population has been exposed to PF4/heparin-like antigens. It is well known that anti-PF4/heparin antibodies are very transient and disappear within 3 months.[9,38] This is a possible reason that the prevalence of anti-PF4/heparin antibodies in the general population (of the SHIP study) does not increase with age. The antibodies found in the normal population rather result from repeated challenge with bacteria, which elicits transient production of the anti-PF4/heparin antibodies. A further interesting finding is the absence of differences between age groups regarding the presence of anti-PF4/heparin IgG and IgM antibodies. This parallels the immune response to blood group A and B, which is also induced by bacteria and in which IgM antibodies are also persistent throughout life despite continuous exposure to the antigen.

We are aware of the limitations of the present study. We could not test whether the antibodies induced in the mice can provoke thrombocytopenia, as platelet activation in HIT is dependent on the presence of the Fc-receptor IIa on platelets and murine platelets do not express this receptor. However, sepsis by itself is causing thrombocytopenia. Even using a transgenic mouse model, which is expressing human PF4 and the Fc-receptor IIa on platelets,[47] it will be extremely difficult to differentiate between sepsis-induced thrombocytopenia and anti-PF4/heparin antibody-induced thrombocytopenia. We also cannot exclude that other strongly negatively charged molecules interact with PF4, thereby exposing the preimmunizing antigen. Thus, the proposed mechanism of preimmunization by PF4-coated bacteria might be just one of several mechanisms.

In conclusion, our experiments indicate that the endogenous chemokine PF4 exposes neoepitopes after charge-related binding to bacteria (Figure 6). This results in the formation of immunogenic clusters of PF4, inducing antibodies with specificity for complexed PF4. These antibodies are able to target a large variety of PF4-coated bacteria and to facilitate their phagocytosis. This indicates a potential new function for PF4, namely, triggering an antibody-mediated immune response against different bacteria. Unfortunately, this antigen is also generated when patients are treated with heparin. Parenteral heparin application leads to binding of heparin to platelets.[48-50] These heparin-coated platelets then expose the PF4/heparin complex antigens, which mimic the epitopes of PF4 complexes formed on bacterial cell surfaces. We propose that HIT represents a misdirected bacterial defense mechanism.

Acknowledgments

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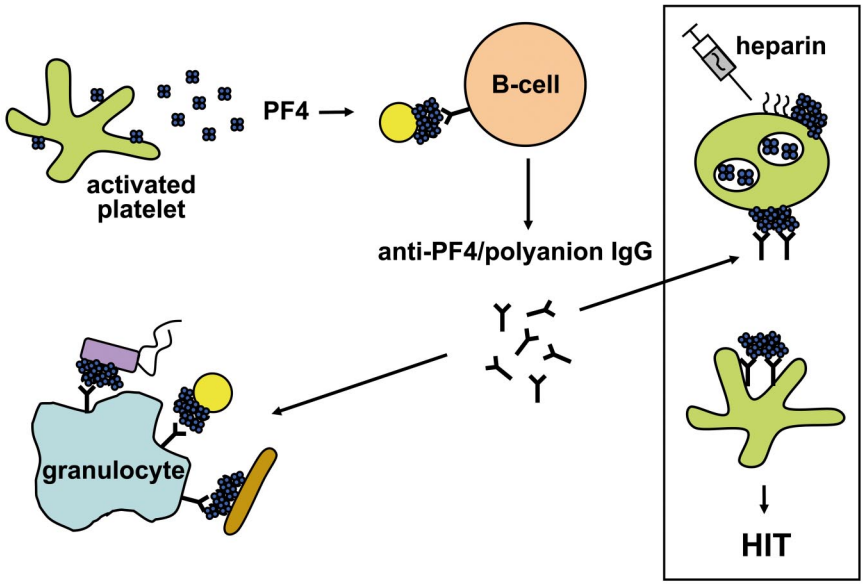

Figure 6. Schematic representation of the mechanism of how PF4 mediates antibacterial host defense and concurrently primes for HIT. Bacteria activate platelets, which release positively charged PF4 interacting with polyanions at the bacterial surface. This generates clusters of PF4, which initiate antibody production by B cells. Once antibodies are induced, they can bind to all bacterial strains, which form PF4 clusters on their surface. Antibody binding to PF4-coated bacteria facilitates binding to granulocytes and subsequently phagocytosis. On the other hand, these antibodies can induce a severe adverse drug reaction, HIT. During heparin treatment, heparin binds to platelets. This mediates formation of PF4/heparin complexes on the platelet surface. These platelet/PF4/heparin complexes mimic PF4 bound to bacteria, and the anti-PF4/polyanion antibodies bind to the platelet surface by their Fab part where they activate platelets by crosslinking the platelet Fc-receptors with their Fc parts. This finally results in massive thrombin generation via a cascade involving activated platelets, platelet microparticles, endothelial cells, and monocytes leading to HIT and new thrombosis. Yellow circles, brown rods, and violet rectangles represent different bacterial species.

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Authorship

Contribution: K.K. designed and performed the in vitro experiments, analyzed the data, and wrote the manuscript; C.P., B.M.B., and W.K. performed the CASP experiments and provided the murine sera; S.M. established the CASP model; B.F. performed the phagocytosis experiments and analyzed the data; S.H. and C.W. provided the bacteria, contributed to the bacterial binding experiments, and revised the manuscript; T.I. evaluated the data of the SHIP study; and A.G. developed the concept, designed the experiments, reviewed and analyzed the results, and wrote the manuscript.

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Comment on Krauel et al, page 1370

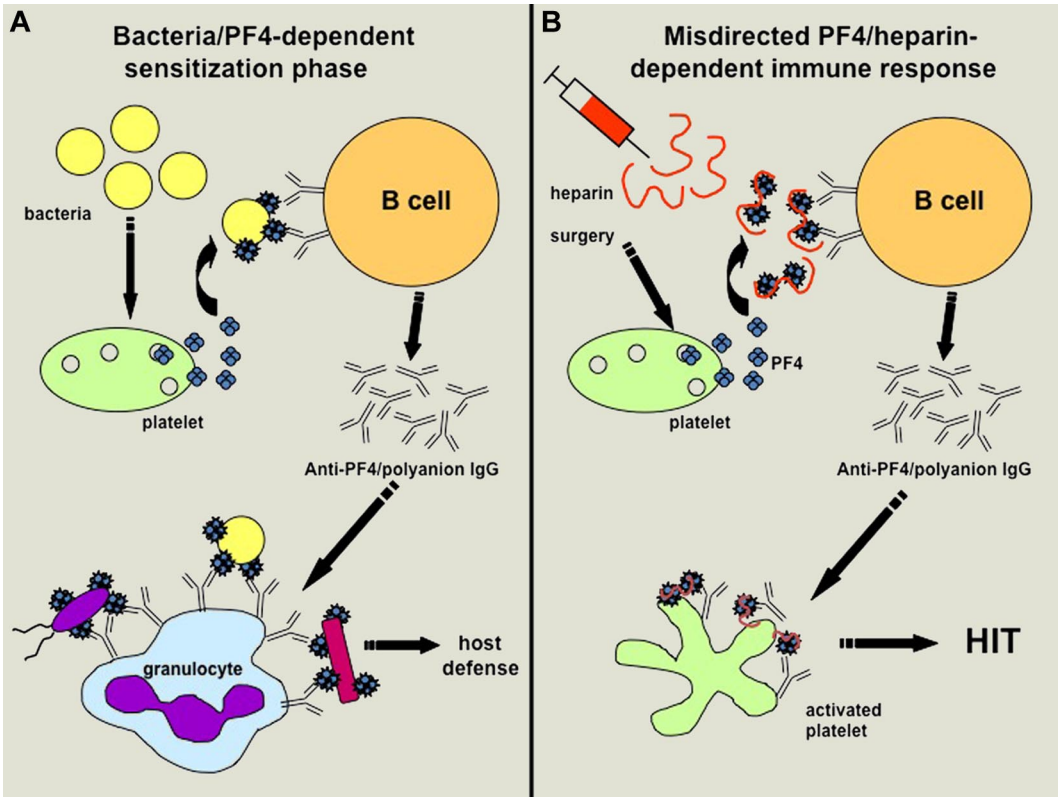
Bacteria and HIT: a close connection?

Yves Gruel and Hervé Watier UNIVERSITÉ FRANCOIS-RABELAIS TOURS

HIT is caused by antibodies specific to PF4/heparin complexes. In this issue of *Blood*, Krauel et al report novel findings supporting the hypothesis that primary synthesis of these antibodies results from bacterial infections. HIT, therefore, appears to be a misdirected antibacterial host defense response.[1]

The immune response to heparin that leads to heparin-induced thrombocytopenia (HIT), a severe drug-induced thrombocytopenia observed in 1% to 3% of heparin-treated patients, is considered to be atypical because IgG and IgM antibodies are both synthesized shortly after heparin administration.[2] HIT IgG antibodies bind to epitopes that are exposed by modified platelet factor 4 (PF4, or CXCL4), a tetrameric member of the C-X-C subfamily of chemokines, after its interaction with heparin. However, a variety of sulfated polysaccharides other than heparin can also induce antigenic changes in the PF4 molecule similar to those implicated in HIT.

In this context, Krauel et al hypothesized that PF4 can also bind to negatively charged polysaccharides present on the surface of bacteria, this interaction leading to the formation of complexes triggering an immune response to modified PF4 that contributes to defense against infections. The results obtained by Krauel et al support this original concept because they showed that human and murine PF4 bound to different strains of Gram-negative and Gram-positive bacteria in a dose-dependent and saturable manner, and this binding was inhibited by high concentrations of heparin. In addition, PF4-coated bacteria could be used to adsorb anti-PF4/heparin antibodies from the sera of patients with HIT, the IgG fractions eluted exhibiting a significant reactivity against PF4/heparin complexes and the ability to activate platelets in the presence of heparin. Using a mouse polymicrobial sepsis model, the authors also demonstrated that bacterial infection was associated with the development of anti-PF4/heparin antibodies, although the mice did not receive any heparin injection. Importantly, the kinetics of the response observed in infected mice resembled a primary immune response with early IgM synthesis followed by relatively low titers of IgG antibodies, which bound to murine PF4 alone. In humans, the specificity of HIT antibodies is mainly directed against PF4/heparin complexes, but IgG may also bind to PF4 alone in some cases. If such a close connection between bacterial infection, PF4, and HIT does exist in humans, we should expect a relatively high prevalence of seroconversion with



Hypothetical representation. The image shows the hypothetical representation of the mechanisms involved (A) in sensitizing infected patients to PF4 and (B) favoring further development of HIT when treated by heparin (adapted from Krauel et al's Figure 6). (A) Bacteria can induce the release of PF4, which then binds polyanions present on the bacterial surface. This interaction triggers the synthesis of antibodies specific to modified PF4, which can bind to variable bacterial strains and favor their phagocytosis by granulocytes. (B) After initiation of heparin treatment (particularly in surgical patients), PF4/heparin complexes are formed, mimic PF4 bound to bacteria and thus trigger the synthesis of modified PF4-specific antibodies in sensitized patients. Anti-PF4/polyanion IgG antibodies strongly activate platelets in some cases and promote the development of HIT.

the development of antibodies to PF4 in patients with sepsis, and this possibility has to be investigated.

Finally, Krauel et al concluded that HIT can be considered as a misdirected host-defense immune response that occurs in some patients exposed to heparin who have been previously immunized with PF4-coated bacteria. This new concept is also supported by the relatively high prevalence of IgM and IgG anti-PF4/heparin antibodies evidenced in a large population of German individuals who had not received any heparin treatment in the 12 months preceding blood sampling. However, it is noteworthy that the same team failed to detect memory B cells in cardiac surgery patients who frequently develop anti-PF4/heparin antibodies postoperatively.[3] To explain this apparent discrepancy, Krauel et al suggest that the immune response associated with HIT could mainly involve marginal zone (MZ) B cells that are major players at the interface between the initial innate immune response and the delayed adaptive response. Indeed, the ability of MZ B cells to respond rapidly to encapsulated bacteria by differentiating into antigen-specific plasma cells helps keep such infections under control. MZ B cells might thus also contribute to the rapid synthesis of IgG, IgM, and IgA PF4-specific antibodies in HIT and explain why there is a rapid decline in antibody titers, which usually disappear within 100 days, whereas a normal memory B-cell response maintains these antibodies for a longer time. The role of T cells in the HIT immune response remains uncertain in this context, although it was supported by a study based on a murine immunization model.[4]

As illustrated in their article (see figure), Krauel et al also deduced from their experiments that antibodies specific to modified PF4 and synthesized in infected patients could contribute to host defenses by allowing the binding of PF4-coated bacteria to granulocytes and their subsequent phagocytosis. Their findings thus support the concept that PF4 has a significant role in bacterial defense. This process could be viewed as a "generic" host-defense mechanism because PF4 was shown to bind to several different strains of bacteria and anti-PF4/polyanion antibodies might therefore react with bacteria not previously encountered by the host-immune system. Whether it is an "ancient" host-defense mechanism, as suggested by Krauel et al, remains to be established. Their findings are also reminiscent of what was observed more than 20 years ago when PF4 was shown to modulate the antibody response to pneumococcal polysaccharides.[5] Moreover, the binding of PF4 to bacteria could also result in direct bactericidal activity dependent on peptides derived from PF4 and containing heparin-binding motifs.[6]

Whether the phenomenon observed by Krauel et al can also occur with other endogenous proteins warrants further study. Intriguingly, peptides derived from another autoantigen, beta-2 glycoprotein I (β2GPI), were recently shown to exhibit antibacterial activities against Gram-positive and Gram-negative bacteria,[7] but it is not known whether the binding of β2GPI to bacteria elicits conformational changes and subsequent anti-β2GPI immune response. However, this protein is the main target of antibodies associated with the "antiphospholipid syndrome" which exhibits several similarities to HIT[8] and can also occur in the context of bacterial infections.

In conclusion, this study is an important contribution that significantly adds to our understanding of the mechanisms potentially responsible for HIT, and opens new areas of research concerning host-defense mechanisms and their links with autoimmunity.

Conflict-of-interest disclosure: The authors declare no competing financial interests. ■

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Comment on Brill et al, page 1400

Clues to DVT pathogenesis

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In this issue of *Blood*, Brill and colleagues demonstrate that von Willebrand factor (VWF) release and subsequent platelet adhesion to endothelial cells are required for thrombus formation in a mouse model of deep venous thrombosis (DVT).

It is probably a good thing that laboratory mice generally do not smoke, use oral contraceptives, become obese, or spend extended periods of time immobilized on long trips, because it turns out that they can indeed develop venous thrombi that greatly resemble human DVTs. The use of laboratory animals to model human disease has been an important component of medical research since at least the mid-19th century when Rudolf Virchow investigated the pathogenesis of pulmonary embolism (PE) using dogs.[1] Interestingly, it was through these studies that Virchow determined that the thrombi found in the lungs actually formed elsewhere in the circulation and somehow traveled to, and became lodged in, the lungs—a process he termed "embolia."[2]

Now more than 150 years later, it is well understood that PEs arise within the deep veins of the lower extremities, and often embolize to the pulmonary circulation with dire physiologic consequences. Despite this process being understood by nearly all physicians worldwide, surprisingly little is known regarding the molecular mechanisms which underlie DVT pathogenesis. Classically, Virchow's triad comprises 3 factors that contribute to venous thrombosis: hypercoagulability,

6.4 Association of natural anti-platelet factor 4/heparin antibodies with periodontal disease

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Association of natural anti-platelet factor 4/heparin antibodies with periodontal disease

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Platelet factor 4 (PF4) and heparin (H) form PF4/H complexes, the target of the immune reaction in heparin-induced thrombocytopenia (HIT). HIT seems to be a secondary immune response as anti-PF4/H-IgG antibodies occur as early as day 4 of heparin treatment. This study investigated whether prevalent infections such as periodontitis may induce the PF4/H immune response as: (1) natural anti-PF4/H Abs are present in the normal population; (2) PF4 bound to bacteria exposes the same antigen(s) as PF4/H complexes; and (3) sepsis induces PF4/H Abs in mice. We found PF4 bound to periodontal pathogens (*Aggregatibacter actinomycetemcomitans; Porphyromonas gingivalis*) enabling subsequent binding of human anti-PF4/H Abs. The association of natural PF4/H Abs and periodontitis was assessed in a case-control study, enrolling individuals with natural anti-PF4/H Abs (n = 40 matched pairs), and in the cross-sectional population-based Study of Health in Pomerania (SHIP; n = 3500). Both studies showed a robust association between periodontitis and presence of anti-PF4/H Abs independent of inflammation markers (case-control study: lowest vs highest tertile, odds ratio, 7.12 [95% confidence interval, 1.73-46.13; *P* = .005]; SHIP study, $p_{trend} \leq 0.001$). Thus, preimmunization to PF4/bacteria complexes by prevalent infections, for example, periodontitis, likely explains the presence of natural anti-PF4/heparin Abs and the early occurrence of anti-PF4/H-IgG in HIT. (*Blood*. 2011;118(5): 1395-1401)

Introduction

The chemokine platelet factor 4 (PF4; CXCL4) is stored within platelet α-granules[1] and released during platelet activation. PF4 binds charge-dependently to polyanions. PF4 has received considerable attention in clinical medicine, as it binds to heparin, thereby forming highly antigenic multimolecular complexes.[2,3] The resulting Ab response[2] induces the most frequent immune-mediated adverse drug reaction involving human blood cells, heparin-induced thrombocytopenia (HIT).[4,5] In HIT, the pathogenic Abs bind to PF4/heparin complexes[3] and the resulting immune complexes induce FcR-mediated platelet activation and enhanced thrombin generation. In a subset of patients this causes thrombocytopenia and triggers thrombosis.[6]

The immune response in HIT is unusual as even patients who receive heparin for the first time generate anti-PF4/heparin-IgG Abs as early as day 4 after start of heparin treatment.[7-9] This indicates preimmunization of patients by means other than heparin treatment.

Recently, we have shown that PF4 also binds to polyanions on cell surfaces of aerobic bacteria forming the same Ag as in PF4/heparin complexes.[10] Accordingly, mice with polymicrobial sepsis generated anti-PF4/heparin Abs.[10] PF4/bacteria complexes are therefore likely candidates for inducing primary immunization which later enables early anti-PF4/heparin-IgG Ab formation during treatment with pharmacologic heparin.

However, sepsis is relatively rare and could not explain why anti-PF4/heparin-IgG Abs are formed in as many as 50% of patients after cardiac surgery within 10 days.[11,12] Furthermore, preimmunization by sepsis could not explain the presence of naturally occurring anti-PF4/heparin Abs in 3%-8% of the normal population.[6,10,13]

We hypothesized that periodontitis might be a cause of preimmunization to PF4/heparin complexes. Periodontitis is one of the most prevalent human infections. The majority of adults suffer from some degree of periodontitis, with 15%-20% of the adult population having severe disease.[14] This inflammatory response to the commensal oral flora is primarily provoked by colonization with anaerobic Gram-negative microbes. The resulting inflammation destroys the tissues that support the teeth (periodontium) forming pockets between the gum and tooth.[15] This destruction can be measured as probing depth. Probing depth equals the distance from the gingival margin to the pocket base and reflects the current inflammatory status of the periodontal disease. In periodontitis, bacteria can invade the blood stream. Bacteremia occurs more frequently in individuals with periodontal disease than in individuals without periodontitis.[16,17] This especially happens during mastication[17] or daily oral hygiene measures.[18,19] Lockhart et al found bacteremia in 28% of individuals immediately after tooth brushing, and in 79% after tooth extraction.[19] It is therefore very likely that bacteremia occurs frequently in individuals with periodontitis.

Periodontitis can also activate platelets by several mechanisms[20] and may thereby trigger release of PF4, which can then bind to bacteria forming PF4/bacteria complexes. To assess our

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hypothesis that periodontal infection may induce a primary immune response to PF4/heparin in humans, we performed in vitro and human studies.

Methods

PF4 binding to periodontal pathogenic bacteria

The major bacterial species associated with periodontitis are *Aggregatibacter actinomycetemcomitans* (*Aa*) and *Porphyromonas gingivalis* (*Pg*).[21] The bacteria used for the PF4 binding study were clinical isolates. The *Aa* (strain Aa1005Rif, clinical isolate) was kindly provided by Helen C. Schreiner (Department of Oral Pathology and Biology, University of Medicine and Dentistry of New Jersey, Newark, NJ) and *Pg* (strain ATCC 33277, clinical isolate) was obtained from the DSMZ-German Resource Center for Biologic Material (Braunschweig, Germany). *Aa* (smooth and rough colonies)[22] was grown in AAGM broth consisting of trypticase soy broth (30 g/L) and yeast extract (6 g/L; 0.4% sodium bicarbonate; 0.8% dextrose; 14 hours; 37°C; 5% CO_2). *Pg* was grown in thioglycollate medium enriched with vitamin K_1 and hemin (Becton Dickinson) in an anaerobic atmosphere (10% CO_2; 37°C; 14 hours; GasPak EZ Pouch System; Becton Dickinson). Bacteria were washed twice with PBS and incubated (30 minutes, 4°C) with biotinylated hu-PF4 (0, 1.25, 2.5, 5, 10, 20, 40 μg/mL). After washing (PBS, 0.05% bovine albumin; 3000*g*, 5 minutes, 4°C), bacteria were incubated (30 minutes; 4°C) with PerCP-Cy5.5 conjugated streptavidin (BD Biosciences), washed again and fixed (1% paraformaldehyde; 20 minutes, 4°C). Binding of PF4 was analyzed by flow cytometry (Cytomics FC 500; Beckman Coulter). The geometric mean fluorescence intensity (GMFI) multiplied by the percentage of labeled bacteria constituted binding activity.

Ab affinity purification

Aa (smooth and rough form) or *Pg* were preincubated with PF4 purified from human platelets (20 μg/5 × 10^7 bacteria, 30 minutes, 4°C; Chromatec) or buffer (PBS, pH 7.4), washed (3000*g*, 5 minutes, 4°C) and incubated (30 minutes, 4°C) with diluted sera of patients known to contain anti-PF4/heparin-IgG Abs (n = 3 per strain), washed to remove unbound Abs, and bound Abs were eluted with glycine buffer (0.1M, pH 2.7; 5 minutes, room temperature) and the supernatant (eluate) neutralized (Tris buffer; 1M, pH 9). The eluates and untreated sera were tested by PF4/heparin-IgG EIA[23] and the heparin-induced platelet activation (HIPA) test.[24]

Periodontal examinations and definitions

For standardization of periodontal examinations, the dental examiner of the case-control study was calibrated against an experienced examiner from the Study of Health in Pomerania (SHIP).[25] In SHIP, calibration exercises were performed parallel to the study each 6-12 months.[25] Probing depth was assessed with a periodontal probe (SHIP: PCP-11, case-control study: PCP-2; Hu-Friedy, Chicago, IL). Mean probing depth was based on all assessed periodontal sites. Extent of probing depth was defined as the percentage of sites equaling or exceeding a certain threshold, as indicated for each analysis; for example, probing depth ≥ 3 mm.

Case-control study

Recruitment of cases and controls. Consecutive blood donors (age 40-60 years) were asked to participate. Exclusion criteria were: < 10 natural teeth; periodontal therapy including dental hygienist treatment within the last 6 months; heparin treatment within the last 3 months. All participants were tested for anti-PF4/heparin Abs of the IgM, IgG, and IgA class by an in-house enzyme-immunoassay (EIA), as previously described.[7,23] Sera were tested with freshly thawed samples twice; reproducible ODs > 0.5, inhibited by high heparin (100 IU/mL) ≥ 40%, were considered positive.

EIA results were reported to an independent study coordinator who invited those testing positive to participate in a periodontal examination. Controls were randomly selected among EIA-negative participants but matched for age (± 2 years), sex, smoking status (never, former, current smoking), and school education (< 10, 10, > 10 years). Participants and the dentist were blinded for the PF4/heparin test result.

SHIP study

SHIP is a population-based prospective study conducted in Western Pomerania, Northeast Germany (details[25,26]). A representative sample of 4308 white subjects (age 20-81 years), randomly drawn from population registries, stratified by age and sex, participated. Information on social demographic characteristics, the medical history, and hospital stays during the last 12 months was gathered via computer-aided personal interviews. Sera were tested once (because of limited material) for anti-PF4/heparin IgG, IgA, and IgM Abs, including a high heparin inhibition step. In addition, high-sensitivity C-reactive protein (hs-CRP, Dade Behring Inc; sensitivity 0.2 mg/L), plasma fibrinogen according to Clauss (Electra 1600 analyzer; Instrumentation Laboratory) and leukocyte counts were determined (Coulter MaxM; Coulter Electronics).

Statistical analyses

Statistical analyses were performed with STATA/SE Version 10 (Stata Corp LP). Categorical data are presented as numbers (percentages), and continuous data are given as median (25th; 75th percentiles). A P value < .05 was considered statistically significant.

Laboratory studies. Differences in reactivities of the eluates were calculated using paired *t* tests.

Case-control study. Paired-sample Wilcoxon signed rank tests were used to detect differences in continuous variables between cases and controls. To evaluate the association between periodontal status (exposure) and anti-PF4/heparin Ab status (dependent variable) conditional logistic regression models with fixed effects were used, adjusting for matching variables.[27] Odds ratios (OR) for anti-PF4/heparin Abs being associated with periodontal disease were determined by exact methods with median-unbiased estimates (mid-P methods).[27] Respective 95% confidence intervals (CI) were reported.

SHIP study. PF4/heparin EIA ODs were analyzed as continuous variables to assess for any potential association between periodontal status and OD reactivity. Differences in the distribution of variables according to quintiles of mean probing depth were analyzed using Kruskal-Wallis (continuous data) and χ^2 tests (categorical data).

The association between periodontal status and anti-PF4/heparin Ab-OD variables was evaluated using linear regression models. Mean probing depth across all measured sites was considered as the main exposure. Analyses were adjusted for age (5-year categories) and sex. Coefficients (B) with their 95% CI were reported. Additional sensitivity analyses were run with the extent of sites presenting a probing depth above a severity threshold as an alternative exposure variable.

The complex sample design was accounted for via survey procedures provided in STATA. Final sampling weights and design variables were used to produce unbiased total estimates.[28]

Ethics

All subjects gave their written informed consent in accordance with the Declaration of Helsinki. The study protocols were approved by the local ethics committee of Greifswald University.

Results

PF4-binding to periodontal bacteria and of anti-PF4/heparin Abs to PF4-coated periodontal bacteria

PF4 bound in a dose-dependent manner to periodontal bacteria (Figure 1A; *Aa* smooth colonies > rough colonies > *Pg*), saturating at 20 μg/mL PF4. Human anti-PF4/heparin Abs bound to PF4-precoated *Aa* and *Pg* (Figure 1B). The IgG Abs eluted from PF4-coated bacteria reacted with PF4/heparin complexes in the

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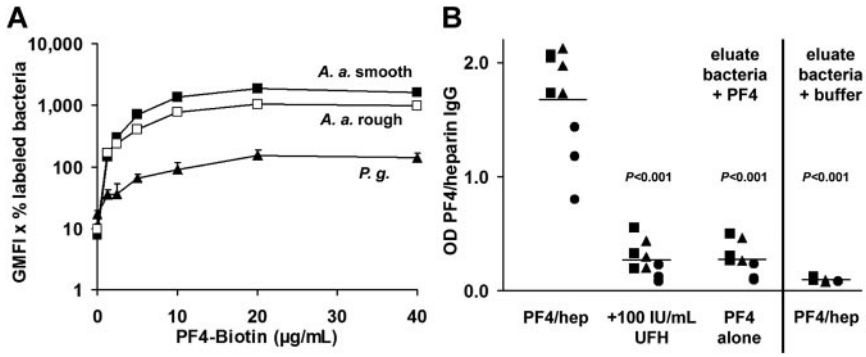

Figure 1. PF4 binds to bacteria found in periodontal lesions and PF4/bacteria complexes are recognized by anti-PF4/heparin IgG from sera of patients with HIT. (A) The anaerobic bacteria *A actinomycetemcomitans* (smooth and rough colonies; *Aa* smooth [n = 4] and *Aa rough* [n = 4]) and *P gingivalis* (*Pg* [n = 5]; both clinical isolates from periodontal pockets) were incubated with human biotinylated PF4. All 3 strains bound PF4 with saturation at approximately 20 μg/mL as measured by flow cytometry and expressed as geometric mean fluorescence intensity (GMFI) multiplied with the percentage of labeled bacteria. Data represent mean ± SD of at least 4 independent experiments. (B) From sera of 3 patients with HIT, known to contain anti-PF4/heparin Abs, IgG Abs were affinity purified using hu-PF4–precoated bacteria. Symbols represent reactivities of Abs affinity purified by PF4-coated *Aa* smooth (■) or rough colonies (▲) and *Pg* (●); means are presented as horizontal lines. Reactivity of the purified Abs with hu-PF4/heparin complexes (first column) was inhibited by excess heparin (100 IU/mL UFH, column 2), which disrupts PF4/heparin complexes. Abs did not react with hu-PF4 alone (column 3). Non-PF4–coated bacteria served as control for unspecific binding of the Abs to bacteria alone (column 4).

PF4/heparin-EIA. This shows that PF4 forms the same Ag(s) when bound to bacteria as it forms when bound to heparin. All controls showed the expected results (Figure 1B): (1) the affinity purified Abs did not react with hu-PF4 alone; (2) excess heparin (100 IU/mL), which disrupts PF4/heparin complexes, inhibited anti-PF4/heparin Ab binding; and (3) non-PF4 coated bacteria did not bind the anti-PF4/heparin Abs.

The anti-PF4/heparin Abs affinity purified from PF4-coated *Aa* (smooth and rough form, 3/3 each) also activated platelets in a functional assay for platelet activating PF4/heparin Abs (HIPA test),[24] at low (0.2 IU/mL) but not at high heparin concentrations (100 IU/mL).

Case-control study

General characteristics and anti-PF4/heparin Ab status. Of 1241 consecutive blood donors, 923 (74.4%) participated. Of those 85 (9.2%) were tested positive; 84 for IgM, one for IgA, and none for IgG. Among those, 40 agreed to undergo periodontal examination. Among 787 negative tested participants, 40 matched controls were recruited (Table 1).

Association between periodontal status and anti-PF4/heparin Ab status. Anti-PF4/heparin Ab positive individuals presented a worse periodontal status compared with the controls; $P < .05$ for all probing depth variables. Periodontal disease severity measured by probing depth was significantly related to anti-PF4/heparin-Ab status (Table 2). Subjects within the highest tertile for mean probing depth had a 7-fold higher risk to be tested positive for anti-PF4/heparin Abs compared with the lowest tertile (OR, 7.12 [95% CI, 1.73-46.13]). Extent values for probing depth > 3, 4, and 5 mm presented similar patterns (Table 2).

Study of health in Pomerania

General characteristics. Among 4308 subjects aged 20-81 years, 3500 (49% men; median age men 52 years, women 49 years) were included in the analysis (missing: 277 anti-PF4/heparin Ab tests; 531 probing depth measurements). Median probing depth was higher in men versus women (2.50 vs 2.27 mm, $P < .001$), highly correlated with age ($r = 0.35$, $P < .0001$), and associated with sociodemographic, behavioral, and laboratory variables (Table 3).

Table 1. Case-control study: characteristics of anti-PF4/heparin Ab negative and positive individuals (matched pairs)

	Anti-PF4/heparin Ab status		
	Negative	Positive	P*
No. of subjects	40	40	
Socioeconomic and behavioral variables			
Male sex	20 (50.0%)	20 (50.0%)	
Age, y	49 (43; 52)	48 (44; 51)	.26
School education			
< 10 y	2 (5.0%)	2 (5.0%)	
10 y	32 (80.0%)	32 (80.0%)	
> 10 y	6 (15.0%)	6 (15.0%)	
Smoking status			
Never smoker	14 (35.0%)	14 (35.0%)	
Former smoker	10 (25.0%)	10 (25.0%)	
Current smoker	16 (40.0%)	16 (40.0%)	
Periodontal variables			
Mean probing depth, mm	2.29 (1.92;2.61)	2.71 (2.35;2.99)	.01
Extent of probing depth ≥ 3 mm, %	30.0 (15.4;58.0)	48.9 (32.9;62.1)	.03
Number of present teeth	25 (22; 26)	23 (21; 26)	.20

Categorical data are presented as numbers (percentages), and continuous data are given as median (25th; 75th percentiles).
*Paired-sample Wilcoxon signed rank test.

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Table 2. Case-control study: exact conditional logistic models regressing anti-PF4/heparin Ab status (dependent variable) on the periodontal status (grouped into tertiles)

	OR (95% CI) for anti-PF4/heparin Abs	*P*
Mean probing depth, mm		
T1 (Ref.)	1.00	–
T2	2.12 (0.56; 10.52)	.28
T3	7.12 (1.73; 46.13)	.005
Extent probing depth ≥ 3 mm, %		
T1 (Ref.)	1.00	–
T2	4.50 (1.28; 22.59)	.02
T3	3.68 (1.02; 18.52)	.05
Extent probing depth ≥ 4 mm, %		
T1 (Ref.)	1.00	–
T2	14.74 (2.77; 370.44)	< .001
T3	14.30 (2.75; 354.53)	< .001
Extent probing depth ≥ 5 mm, %		
T1 (Ref.)	1.00	–
T2	5.54 (1.40; 32.91)	.01
T3	6.32 (1.87; 31.82)	.002

ORs for the presence of anti-PF4/heparin Abs with their 95% CI (median-unbiased, mid-P-method) and *P* values are given; n = 40 matched pairs. Models were adjusted for age (continuous), sex, school education, and smoking status. Tertiles for mean probing depth were: ≤ 2.24 mm, 2.25-2.73 mm, and 2.75-4.75 mm; for extent probing depth ≥ 3 mm: ≤ 27.3%, 27.5%-52.3%, and 55.5%-89.3%; for extent probing depth ≥ 4 mm: 0%-2.3%, 2.5%-22.9%, and 23.1%-68.8%; for extent probing depth ≥ 5 mm: 0%, 1.8%-5.6%, and 5.8%-53.8%.

OR indicates odds ratio; CI, confidence interval; T, tertile; and –, not applicable.

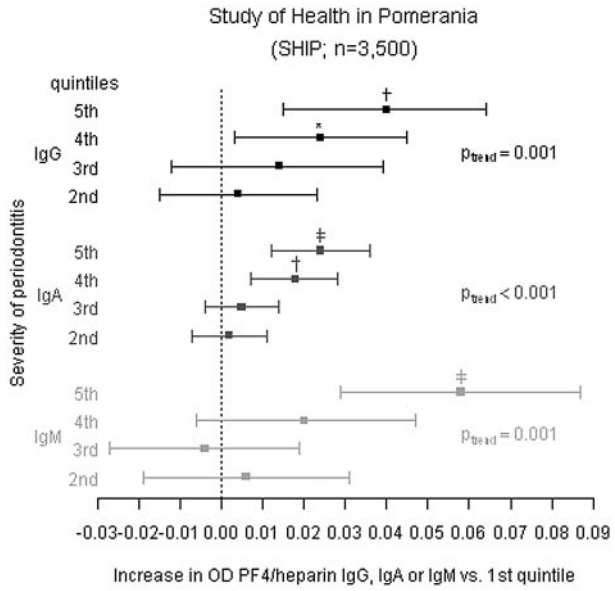

Figure 2. The ODs in the PF4/heparin EIA depend on the periodontal status in non–heparin-treated individuals. In 3500 individuals of the SHIP cohort the periodontal status (assessed by probing depth) and anti-PF4/heparin Ab EIA ODs for IgG, IgA, and IgM were determined. The individuals were grouped into quintiles according to their mean probing depth. Difference in mean ODs in the anti-PF4/heparin EIA (squares) and 95% CIs are given for quintiles 2 to 5. The individuals in the first quintile were used as reference group. Linear regression models were adjusted for age (5-year categories) and gender (reported as M1 in Table 4). P_{trend} indicates p for linear trend across quintiles. *$P < .05$; †$P < .01$; ‡$P < .001$ compared with the first quintile. Additional linear regression analyses are given in Table 4.

Relationship between probing depth and anti-PF4/heparin Ab status. After adjusting for age and sex, mean probing depth was significantly linearly related to the reactivity of the sera in the PF4/heparin EIA as measured by OD (Figure 2, Table 4, M1). On average, mean anti-PF4/heparin OD values increased per quintile compared with the reference quintile for IgG ($P_{trend} = .001$), IgA ($P_{trend} < .001$), and IgM ($P_{trend} = .001$). All findings were consistent if subjects were restricted to those without hospital stays during the last 12 months (Table 4 M2).

Table 3. SHIP: population characteristics (n = 3500)

	Mean probing depth, mm					
	Q1 (1.04-1.95 mm)	Q2 (1.95-2.25 mm)	Q3 (2.25-2.53 mm)	Q4 (2.54-3.00 mm)	Q5 (3.02-9.25 mm)	*P*
No. of subjects	701	761	638	750	650	
Socioeconomic status						
Age, y	41 (31; 56)	48 (36; 60)	53 (40; 64)	56 (46; 65)	71 (64; 75)	< .001
Male sex (%)	276 (39.4%)	309 (40.6%)	309 (48.4%)	419 (55.9%)	402 (61.9%)	< .001
School education (%)						
< 10 y	121 (17.2%)	193 (25.4%)	211 (33.1%)	323 (43.1%)	368 (56.6%)	
10 y	377 (53.8%)	423 (55.6%)	300 (47.0%)	333 (44.4%)	232 (35.7%)	
> 10 y	203 (29.0%)	145 (19.0%)	127 (19.9%)	94 (12.5%)	50 (7.7%)	< .001
Smoking status (%)						
Never smoker	273 (39.0%)	307 (40.5%)	256 (40.1%)	231 (31.0%)	176 (27.3%)	
Former smoker	221 (31.5%)	224 (29.5%)	222 (34.8%)	263 (35.2%)	212 (32.8%)	
Current smoker	207 (29.5%)	227 (30.0%)	160 (25.1%)	252 (33.8%)	258 (39.9%)	< .001
Hospital stays during last 12 months (%)						
Yes	96 (13.7%)	100 (13.2%)	85 (13.3%)	128 (17.1%)	85 (13.1%)	
No	605 (86.3%)	657 (86.8%)	553 (86.7%)	620 (82.9%)	561 (86.7%)	
Refused answer	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1.2%)	.18
Laboratory measurements						
hs-CRP, mg/L	0.9 (0.4; 2.2)	1.1 (0.5; 2.5)	1.3 (0.6; 2.8)	1.4 (0.7; 3.2)	1.8 (0.8; 3.9)	< .001
Leukocytes, Gpt/L	6.1 (5.2; 7.4)	6.2 (5.2; 7.7)	6.3 (5.4; 7.4)	6.6 (5.4; 7.9)	6.8 (5.7; 8.3)	< .001
Fibrinogen (Clauss), g/L	2.3 (2.7; 3.1)	2.8 (2.4; 3.2)	2.8 (2.5; 3.3)	2.9 (2.5; 3.5)	3.0 (2.7; 3.6)	< .001
Periodontal measurements						
No. of teeth	22 (25; 28)	24 (20; 26)	23 (18; 26)	20 (13; 25)	15 (8; 21)	< .001

Categorical data are presented as numbers (percentages), and continuous data are given as median (25th; 75th percentiles). The number of subjects with additionally missing data was n = 9 for hospital stays during last 12 months, n = 58 for hs-CRP, n = 2 for leukocytes, and n = 9 for fibrinogen (Clauss).

SHIP indicates Study of Health in Pomerania; Q, quintile; and hs-CRP, high-sensitive C-reactive protein.

*Kruskal-Wallis-test (continuous data) or χ^2 test (categorical data).

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Table 4. SHIP: association between mean probing depth (quintiles) and anti-PF4/heparin-ELISA ODs assessed with linear regression

	Mean probing depth, mm					
	Q1 (1.04-1.95 mm)	Q2 (1.95-2.25 mm)	Q3 (2.25-2.53 mm)	Q4 (2.54-3.00 mm)	Q5 (3.02-9.25 mm)	*P* for linear trend
IgG						
M1	0.000	0.004 (−0.015; 0.023)	0.014 (−0.012; 0.039)	0.024 (0.003; 0.045)*	0.040 (0.015; 0.064)†	.001
M2	0.000	0.002 (−0.018; 0.022)	0.015 (−0.013; 0.042)	0.022 (−0.001; 0.044)	0.044 (0.017; 0.070)†	.001
M3	0.000	0.004 (−0.015; 0.023)	0.014 (−0.011; 0.039)	0.024 (0.003; 0.045)*	0.037 (0.012; 0.061)†	.002
M4	0.000	0.003 (−0.014; 0.019)	0.007 (−0.015; 0.029)	0.021 (0.003; 0.040)*	0.041 (0.018; 0.064)†	< .001
IgA						
M1	0.000	0.002 (−0.007; 0.011)	0.005 (−0.004; 0.014)	0.018 (0.007; 0.028)†	0.024 (0.012; 0.036)‡	< .001
M2	0.000	−0.000 (−0.010; 0.009)	0.004 (−0.006; 0.014)	0.016 (0.004; 0.027)*	0.024 (0.012; 0.037)‡	< .001
M3	0.000	0.001 (−0.008; 0.010)	0.004 (−0.005; 0.014)	0.016 (0.005; 0.027)†	0.022 (0.010; 0.035)†	< .001
M4	0.000	0.001 (−0.008; 0.010)	0.005 (−0.005; 0.014)	0.015 (0.005; 0.026)†	0.024 (0.012; 0.036)‡	< .001
IgM						
M1	0.000	0.006 (−0.019; 0.031)	−0.004 (−0.027; 0.019)	0.020 (−0.006; 0.047)	0.058 (0.029; 0.087)‡	.001
M2	0.000	0.002 (−0.025; 0.029)	−0.000 (−0.026; 0.025)	0.013 (−0.017; 0.043)	0.061 (0.029; 0.093)†	.002
M3	0.000	0.003 (−0.022; 0.028)	−0.009 (−0.033; 0.014)	0.013 (−0.014; 0.039)	0.051 (0.022; 0.081)†	.004
M4	0.000	0.001 (−0.022; 0.024)	−0.002 (−0.025; 0.020)	0.017 (−0.008; 0.042)	0.059 (0.030; 0.087)‡	< .001

Coefficients with 95% CI are presented (n = 3500). M1: adjusted for age (5-year categories) and sex. M2: M1, excluding hospital stays during the last 12 months (n = 494 subjects excluded). M3: M1, plus hs-CRP, leukocytes and fibrinogen (Clauss) included as quintiles (n = 68 subjects with missing data excluded). M4: M1, PF4/heparin EIA OD > 0.5 and OD inhibited > 40% by high heparin (n = 106 subjects excluded).

SHIP indicates Study of Health in Pomerania; Q, quintile; CI, confidence interval; and hs-CRP, high-sensitive C-reactive protein.

*$P < .05$; †$P < .01$; and ‡$P < 0.001$ compared to the first quintile.

Models additionally adjusted for inflammatory parameters (quintiles of hs-CRP, leukocytes, and fibrinogen) barely changed estimates across probing depth quintiles (Table 4 M3). Restriction to subjects with anti-PF4/heparin Ab ODs > 0.5 and inhibition of > 40% by high heparin did also not change results markedly (Table 4 M4). Substituting mean probing depth by the extent of probing depth above varying severity thresholds (≥ 3, 4, 5 mm) confirmed the results (supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Number of teeth was not associated with the presence of anti-PF4/heparin Abs.

Discussion

In this study, we provide several lines of evidence that periodontitis may play a role in the immune response to PF4/heparin complexes. First, PF4 binds to periodontal bacteria. Second, PF4 bound to these bacteria exposes the same Ag as induced by PF4/heparin complexes. This allowed affinity purification of heparin-induced human anti-PF4/heparin Abs using PF4-coated periodontal bacteria. These in vitro findings are substantiated by 2 clinical studies, both showing highly significant associations between the severity of periodontal disease and the presence of natural anti-PF4/heparin Abs. The dose-response relationship between the severity of periodontal disease, which is characterized by an increasing burden of microbes in the periodontal pocket, and the frequency of natural anti-PF4/heparin Abs makes it even more likely that primary immunization to PF4/heparin complexes is induced by bacterial infection(s).

We recently hypothesized that the anti-PF4/heparin Ab response represents an evolutionary ancient host-defense mechanism by which Abs against PF4/heparin complexes can recognize a wide variety of bacteria when this chemokine is bound to them. Moreover, we have shown that phagocytosis of anti-PF4/heparin Ab-coated bacteria is enhanced.[10] It would be plausible that such a defense mechanism is present in the mouth were the exposure to bacteria is especially high.

In the ulcerated periodontal pockets, the relatively harmless oral bacteria gain access into the highly vascularized granulation tissue adjacent to the teeth and then into the blood circulation.[16,17,29] This everyday occurrence is thought to be one of the key initiators of biologic events which make oral infections an important risk factor for cardiovascular disease, independent of traditional cardiovascular risk factors.[30] In fact, common periodontal pathogens have been recovered from carotid endarterectomy plaque samples.[31-33] To reach the atherosclerotic plaques, bacteremia must have occurred. Interestingly, PF4 has also been found in these atherosclerotic plaques.[34] Whether anti-PF4/heparin Abs interact with leukocytes or platelets in these plaques and potentially even increase local inflammation requires further studies.

One limitation of our study is that we did not quantify the periodontal bacteria either in the pockets nor in the blood. However, the association between subgingival bacterial burden and probing depth is well described in the literature. Demmer et al showed that the severity of periodontal infection (quantified by clinical periodontal measures as in our studies) correlates with bacterial burden, reporting a close correlation (r = 0.61) between the number of sites with probing depth > 3 mm with the subgingival bacterial burden.[35] Paju et al reported an association between the number of teeth with deepened pockets and carriage of periodontal pathogens.[36]

For binding of PF4 and induction of the PF4/heparin immune response, the bacteria have to enter the blood stream. Previous studies already showed that the incidence and intensity of bacteremia correlate with the extent and severity of periodontitis.[16,37] It is very difficult to quantify the amount of bacteria that initially gain entrance to the circulation after dental procedures. In volunteer studies, quantification of bacteria in the blood can only be made from venous blood taken from the arm vein. Thus, the blood from the gums has to circulate at least once through the body before it reaches the cubital vein. The periodontal bacteria are diluted by the blood volume and likely most bacteria are already cleared during the first passage in the liver. Furthermore the detection of periodontal Gram-negative anaerobic bacteria is technically very demanding. To overcome the problem of bacterial enumeration and detection Geerts et al quantified the endotoxin load as a proxy for the magnitude of the Gram-negative bacteremia induced by gentle mastication.[17] They found endotoxinemia in 6% of individuals

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before and in 24% after mastication with especially high levels in individuals with severe periodontal disease. Taken together, all these studies unequivocally showed bacteremia occurring regularly in subjects with periodontal disease.

It is well known that anti-PF4/heparin Abs are very transient and disappear within 3 months,[7,8] probably because no memory B cells with PF4/heparin specificity are formed.[38] We regard this as a very likely reason that the prevalence of anti-PF4/heparin Abs in the general population is not that high. In our opinion the Abs found in the normal population are rather the result from repeated challenges with bacteria which elicits transient production of the anti-PF4/heparin Abs. In this regard, the differences in prevalence of anti-PF4/heparin IgG Abs in the case control study and the cross-sectional population study are of interest. We have previously shown that PF4/heparin Ab formation also depends on patient related factors, such as trauma or inflammation.[39] We also speculated before that a special B-cell population, probably marginal zone B cells, might be involved in the immune response of HIT.[7] These B cells seem to require a second stimulus, like trauma or inflammation, to produce PF4/heparin Abs, especially of the IgG class. The blood donor population of our case control study had periodontal disease but otherwise they were preselected, healthy regular blood donors. In contrast, the individuals of the cross-sectional population study were also included if comorbidities were present, such as diabetes, poorly controlled hypertension, allergies, bowel disorders, etc. This might be one reason for the low prevalence of anti-PF4/heparin IgG Abs in the blood donor cohort. In line with this hypothesis, we also recently found[40] a higher prevalence of anti-PF4/heparin Abs in medical patients at hospital admission and before heparin treatment, compared with blood donors.

We addressed the question whether anti-PF4/heparin Abs are potentially only an epiphenomenon of inflammation and thus an inflammation marker rather than a specific immune reaction. To evaluate this, we included inflammatory markers (hs-CRP, leukocyte counts, and fibrinogen level) known to be increased in periodontal disease[41,42] in the regression models (see Table 4 M3). If the association would have been mostly mediated via systemic inflammation, integration of these variables should shift regression coefficients of the exposure (periodontitis assessed by quintiles of mean probing depth) to the Null. However, including inflammatory markers in the multivariate regression model did not attenuate regression coefficients, supporting the assumption that the association was not or only to a minor part mediated by systemic inflammation. This strengthens our hypothesis that the occurrence of natural anti-PF4/heparin Abs may be triggered directly via periodontal pathogens rather than indirectly via systemic inflammation.

Our study has further limitations. The bacteria used for adsorption-elution studies were clinical isolates but not obtained from the immunized individuals themselves and not all these bacteria might have the same binding capacity for PF4. There might also be other, yet unknown, routes of bacterial infection to induce these Abs. We could not assess whether the Abs found in the cross-sectional population study were platelet activating because of lack of material and we do not have follow-up data on whether treatment of periodontal disease lowers the incidence of the anti-PF4/heparin Abs. These questions warrant further studies, given that formation of natural anti-PF4/heparin Abs might represent a potential pathway that could explain the epidemiologic association between cardiovascular morbidity and mortality in subjects with periodontal disease.[30]

In conclusion, PF4 binds to bacteria typically found in periodontal disease hereby exposing the Ag(s) recognized by human anti-PF4/heparin Abs, and the severity of periodontal disease correlates with the presence of anti-PF4/heparin Abs in otherwise healthy individuals. Preimmunization to PF4/bacteria complexes, caused by prevalent infections such as periodontitis, might therefore explain the presence of natural anti-PF4/heparin Abs and the occurrence of anti-PF4/heparin-IgG Abs as early as day 4 in patients treated with heparin for the first time.[8]

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Authorship

Contribution: A.G. and T.K. designed the study, analyzed and interpreted the data, and wrote the manuscript; K.K., C.W., and S.H. performed the in vitro experiments, designed the bacteria experiments, and wrote the manuscript; B.H. and T.I. designed the statistical part of the study, analyzed the data, and wrote the manuscript; D.G. performed the dental examinations of the case-control study; and all co-authors critically revised the manuscript and approved the final version.

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7 Anhang

7.1 Zusammenfassung

Plättchenfaktor 4 (PF4, CXCL4) ist ein 7.8 kDa stark positiv geladenes Protein, das zur Familie der CXC-Chemokine gehört. PF4 wird als Tetramer in den alpha-Granula von Thrombozyten gespeichert und bei deren Aktivierung freigesetzt. Seine biologische Funktion ist weitgehend unbekannt. Allerdings spielt PF4 eine wichtige Rolle bei der Heparin-induzierten Thrombozytopenie (HIT), einer der häufigsten immunologisch bedingten Arzneimittelkomplikationen, die Blutzellen betreffen. PF4 und Heparin bilden Komplexe, die Neoepitope generieren und die Produktion von anti-PF4/Heparin-Antikörpern induzieren. Resultierende Immunkomplexe aktivieren Thrombozyten Fc-Rezeptor-vermittelt intravasal und führen zu einer paradoxen Thrombinbildung. Die Folgen können Thrombozytopenie und lebensbedrohliche Thrombosen sein. Diese pathogenen Antikörper binden an PF4. Heparin ist „nur“ die Quelle für linear angeordnete negative Ladungen, die eine Komplexierung von mehreren PF4-Tetrameren erlaubt. Heparin kann hierbei durch andere lineare Polyanionen ersetzt werden. Die Immunantwort der HIT zeigt einige Besonderheiten. Bereits bei erstmaliger Heparinexposition treten anti-PF4/Heparin-Antikörper der Klasse IgG bereits nach 4-6 Tagen auf. Dies kann keine primäre Immunantwort sein, da bei dieser IgG-Antikörper erst deutlich später gebildet werden. Damit muss eine Vorimmunisierung mit natürlich auftretenden PF4/Polyanion-Komplexen stattgefunden haben. Eine mögliche Quelle wären negativ geladene Strukturen, die auf bakteriellen Zelloberflächen vorkommen.
Ziel dieser Arbeit war es, die Bindung des Thrombozytenproteins PF4 an Zelloberflächen zu charakterisieren und insbesondere die Frage zu klären, ob PF4 auf der Bakterienoberfläche Komplexe bildet, die eine Immunisierung gegen PF4/Heparin-Komplexe erklären könnte. Zunächst erfolgte die Etablierung einer durchflusszytometrischen Analysemethode zur Messung der PF4-Bindung an Thrombozyten in Abhängigkeit von antikoagulatorischen Polyanionen. Die PF4-Bindung war nicht von der antikoagulatorischen Wirksamkeit der Polyanionen abhängig, sondern von der negativen Ladungsdichte. Geringe Konzentrationen von Heparin haben die PF4-Bindung an Thrombozyten verstärkt, wohingegen hohe Konzentrationen die PF4-Bindung inhibierten. Auch mit PF4 exprimierenden HEK-Zellen konnte eine ladungsabhängige Bindung von PF4 nachgewiesen werden. Als nächstes wurde die Bindung von PF4 an Bakterien untersucht. PF4 hat auch hier, konzentrations- und ladungsabhängig, an Gram-positive und Gram-negative Bakterien gebunden. Mittels der

Adsorptions-Elutions-Technik konnte dann gezeigt werden, dass es durch die Bindung von PF4 an Bakterien zur Ausbildung PF4/Heparin-ähnlicher Epitope kommt, welche von anti-PF4/Heparin-Antikörpern aus Patientenserum erkannt werden. Mit einem Phagozytoseassay wurde dann nachgewiesen, dass die durch PF4 vermittelte Bindung der anti-PF4/Heparin-Antikörper an Bakterien die Phagozytose durch polymorph nukleäre Zellen (PMN) fördert. Dass dieser Abwehrmechanismus tatsächlich *in vivo* bedeutsam ist, konnte in dem Maus-Sepsis-Modell Colon Ascendens Stent Peritonitis (CASP) gezeigt werden. Die 6-8 Wochen jungen Mäuse entwickelten ab 3 Tagen nach der CASP-Operation anti-PF4/Heparin-Antikörper der Klasse IgM und ab 14 Tagen der Klasse IgG. Dieser Antikörperverlauf passt zur Immunreaktion nach erstmaligem Antigenkontakt und beweist, dass Bakterienkontakt im Rahmen der Sepsis eine primäre Immunisierung gegen PF4/Heparin induzieren kann. Jedoch ist die Sepsis, eine lebensbedrohliche Erkrankung, viel zu selten um das Auftreten von anti-PF4/Heparin-Antikörpern in der Normalbevölkerung (18.8% anti-PF4/Heparin IgM, 6.1% anti-PF4/Heparin IgG) und bei 50% der Patienten nach kardiochirurgischem Eingriff erklären zu können. Eine häufiger auftretende, chronische, bakterielle Infektion ist die Parodontitis. Im Rahmen der SHIP-Studie (Study of Health in Pomerania) konnte dann gezeigt werden, dass der Parodontitis-Status mit dem Auftreten von anti-PF4/Heparin-Antikörpern korreliert. Unterstützend konnte gezeigt werden, dass PF4 auch von parodontalpathogenen Bakterien gebunden wird und darüber die Bindung von anti-PF4/Heparin-Antikörpern ermöglicht wird.
Zusammenfassend zeigt diese Arbeit eine bislang unbekannte Funktion von PF4 in der antibakteriellen Wirtsabwehr. Gleichzeitig wurde die Frage geklärt, warum Patienten, die das erste Mal Heparin erhalten bereits an Tag 4-6 IgG-Antikörper gegen PF4/Heparin bilden. Somit konnte ein wichtiger Beitrag zur pathophysiologischen Erklärung einer der derzeit häufigsten immunvermittelten, unerwünschten Arzneimittelwirkungen geleistet werden.
Hinsichtlich der biologischen Bedeutung von PF4 ist von besonderer Wichtigkeit, dass PF4 ladungsabhängig an viele verschiedene Bakterienspezies bindet. Damit sind Antikörper gegen PF4/Polyanion-Komplexe nicht spezifisch für eine Bakterienspezies, sondern können eine Form der Wirtsabwehr darstellen, bei der eine IgG-Spezifität eine Vielzahl von PF4-markierten Bakterien bindet. Es wäre denkbar, dass es sich hierbei um ein grundlegendes, evolutionär altes Abwehrprinzip handelt, dass möglicherweise auch bei anderen immunvermittelten Erkrankungen Relevanz hat.

7.2 Wissenschaftliche Leistungen

7.2.1 Veröffentlichungen

K. Krauel, C. Hackbarth, B. Fürll, and A. Greinacher. Heparin-induced thrombocytopenia: in vitro studies on the interaction of dabigatran, rivaroxaban, and low-sulfated heparin, with platelet factor 4 and anti-PF4/heparin antibodies. Blood. 2011. Epub ahead of print.

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7.2.2 Kongressbeiträge

C. Weber, K. Krauel, A. Greinacher, and S. Hammerschmidt. 2011. Lipopolysaccharides of Gram-negative and teichoic acids and proteins of Gram-positive bacteria contribute to binding of platelet factor 4 to bacteria facilitating the creation of heparin-induced thrombocytopenia-eliciting antibodies. 63. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM), Essen, Deutschland. (Poster)

K. Krauel**,** C. Hackbarth, B. Fürll, and A. Greinacher. 2011. Low sulfated heparin (ODSH) interferes with anti-PF4/heparin antibody induced platelet activation by disrupting PF4/heparin complexes. XXIII Congress of the International Society on Thrombosis and Haemostasis (ISTH), Kyoto, Japan. (Poster)

K. Krauel**,** C. Hackbarth, B. Fürll, and A. Greinacher. 2011. Dabigatran does not interact with PF4 or PF4 binding to platelets and might be used as an alternative anticoagulant in heparin-induced thrombocytopenia. XXIII Congress of the International Society on Thrombosis and Haemostasis (ISTH), Kyoto, Japan. (Vortrag)

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7.3 Eidesstattliche Erklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Greifswald, den 26.01.2012 K. Krauel

7.4 Lebenslauf

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Ausbildung

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