Identification and Characterization of Anti-Platelet Antibodies in Idiopathic Thrombocytopenic Purpura Patients

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Abstract

Background: The autoimmune disease known as Idiopathic (immune thrombocytopenic purpura thrombocytopenic purpura (ITP) is clinically defined by a low numbers of platelets in the circulation blood. This study aimed to isolate autoantibodies made against the platelet glycoproteins using platelets from healthy volunteers, to determine their specificity and further elucidate their effects on platelet function.

Methods: This study used a phage display system to recognize Fab anti-platelet antibodies. Anti-platelet After isolation, the anti-platelet Fab-expressing phage was characterized by ELISA and Western blotting. The Fab-bearing phage pool obtained from five rounds of panning was analysed in order to determine its anti-platelet reactivity. Of the phage colonies obtained, 100 colonies of different sizes were randomly selected for reaction with whole platelets, using M13 phage as a negative control.

Results: Twelve colonies of them had strong reactions against the whole platelet preparation, but only four colonies showed substantial reactivity against the lysed platelet preparation (lysate). Three of the four colonies showed three bands representing proteins with different molecular weights. The fourth colony showed only a single band. The final experiment to characterise the protein isolated from the phage library was a DNA gel agarose test.

Conclusion: Each colony showed a DNA band that corresponded with the molecular size marker for 5.4 kbase pairs, and this suggested the presence of heavy and light antibody chains in the phage.

Keywords: Idiopathic Thrombocytopenic Purpura, Platelet, Antibody

Introduction

Idiopathic (immune) thrombocytopenic purpura (ITP) is an autoimmune disease that involves the development of autoantibodies against platelet cells in the blood, which play an essential role in the haemostatic control of bleeding. There are estimated to be 10 to 125 new cases of ITP per million of the population every year (1). In the USA, annual rates for adults are approximately 66 cases per million and for children are approximately 50 cases per million. There are about 10 cases of chronic refractory ITP per million per year. Figures from outside the USA show approximately 10-40 cases in children per mil-

lion per year in Denmark and the UK and approximately 125 cases per million per year in Kuwait (2). In a report which added to evidence obtained since the 1960s that the proportion of black people with ITP was lower than the proportion of black people in the general population of the United States, and that the worldwide occurrence of ITP is rare among black Africans and people of African ancestry (3-8).

Around half of the 50–100 new cases seen per million people per year are in children, and this tends to be the acute form (platelet counts of less

than 27,000 per µl), especially in children younger than 10 years old. The chronic form (platelet counts of over 27,000 per µl) is generally seen in older patients (9). There was only a slight difference between boys (55%) and girls (45%) (10). Age over 10 years is a risk factor for developing the chronic form of the disease (11). Thrombocytopenia refers to low levels of platelets in the circulation, from any cause. A normal (healthy) count is somewhere between 150,000 and 450,000 platelets per µl of blood.

The main clinical sign of ITP is purpura. The bleeding tendency in ITP is caused by a platelet disorder (12). Because it involves a platelet disorder, ITP is known as a thrombocytopathy (13) and because it involves a decrease in platelet function it is also known as a thrombasthaenia.

ITP is characterised by the premature immune destruction of platelets, with production of autoantibodies against platelets, specifically against the glycoprotein complexes in the platelet membrane, such as GP IIb/IIIa or GP Ib/IX. The glycoprotein complexes are physiological receptors that mediate interactions between platelets and vascular subendothelium in injured blood vessels. By their adhesion and aggregation activities, the platelets arrest haemorrhaging from lesions in the blood vessel wall, and any disruption to their membrane glycoproteins leads to platelet dysfunction and destruction, and causes the bleeding disorders seen in ITP. Our understanding of the autoimmune nature of the destruction of peripheral platelets in ITP dates back to the 1950s (14-15), but to appreciate this complex phenomenon the functioning of the immune system needs to be explained fully. This study aimed to isolate autoantibodies made against the platelet glycoproteins using platelets from healthy volunteers, to determine their specificity and further elucidate their effects on platelet function. As there is no accurate diagnostic test for ITP, new evidence about these autoantibodies may be of great value in the future.

Materials and Methods

Sixty milliliter anti-coagulated (ACD) blood, prepared from healthy volunteer (PhD student) was centrifuged at 200 g for 20 minutes at room temperature (15-22 ^{0}C). After which the platelet rich plasma (PRP) was removed. 1 µg/ml diluted prostaglandin (1 in 4 in ethanol) was added and the PRP re-centrifuged at 1200 g for 12 minutes. After washing the sedimented platelets four times with isotonic citrate buffer, the platelets were resuspended in 1 ml isotonic buffer containing 10% dimethylethyl sulfoxide (DMSO) and aliquoted at a concentration of 10^9 ml^{-1} and stored at -20 °C. The thawed platelets were washed with isotonic citrate buffer before use. The concentrated platelet proteins were extracted from Purified whole platelets. Monoclonal antibodies anti human CD41 (GP IIb/IIIa) and anti human CD-61 (GP IIIa) (Novacastra Company Ltd) were used to detected platelet membrane glycoproteins by ELISA method (Novacastra Company protocol). Preparation of electrocompetent *Escherichia coli* (K12) from a glycerol stock and then adding the commercial helper phage (VCSM13; Stratagene) to prepare M13 helper phage were done by using standard culture media and method (Stratagene). Alkaline lysis releases plasmid DNA from bacteria and ribonuclease A (RNase A) removes all the RNA in the lysate. Plasmid DNA is purified using HighPure^{TM} plasmid isolation kit. 100 µl of electrocompetent *Escherichia coli* cells (K12) were inoculated into a pre-chilled tube with 5 µl of plasmid DNA (from a phage library containing DNA of the heavy chain domains VH and CH1, and light chain domains VL and CL, of the antibodies in PAK100 vector, constructed using mRNA obtained from splenic lymphocytes of one patient with idiopathic thrombocytopenic purpura (ITP) and systemic lupus erythematosus (the phage library was a gift from Dr Lynda Partridge at the University of Sheffield). To confirm whether the DNA of the heavy and light chains had been inserted into the plasmid DNA,

the DNA was cut with restriction enzymes to isolate the insertion. For each sample, three Eppendorf tubes were prepared: one for the heavy chain digest, one for the light chain digest, and one for the undigested DNA. Then 25 µl DNA was added to each tube, along with 2 µl of the restriction enzymes.BstXI (10 U/µ1) and Xhol XI (10 U/µl) were used for the heavy chain, 2 µl of XbaI (10 U/µl) and 2 µl SacI (10 U µl-1) were used for the light chain, and 2 µl of Nehl (10 U/µl) was used for the undigested sample.

The use of the polymerase chain reaction (PCR) to produce a large number of identical copies of DNA sequences is useful for amplifying the gene segments encoding the V domains of antibody (16). The gene to be replicated is inserted into copies of a plasmid containing genes that make cells resistant to particular antibiotics and a multiple cloning site. The plasmids are then inserted into bacteria by a process called transformation. When the bacteria are exposed to particular antibiotics, only the bacteria that take up copies of the plasmid survive, because the plasmid makes them resistant. The protecting genes are expressed (used to make a protein) and the expressed protein breaks down the antibiotics. In this way the antibiotics act as a filter to select only the modified bacteria (17). These bacteria can be grown in large amounts, then harvested and lysed (often using the alkaline lysis method) to isolate the plasmid of interest. Antibody specific for a particular antigen can be selected from the library by panning. The phage that binds is recovered and amplified through used to infect fresh *Escherichia coli*.

A single-chain variable fragment (scFv, 5×10^5) phagemid library of human immunologlobulin light (L) and heavy (H) chains was constructed in this study. The mRNA from the splenic lymphocytes of a patient with ITP in the vector PAK 100 was used (18). This patient suffered from both SLE and ITP. The phage library was a gift from Dr Lynda Partridge from the University of Sheffield. Glycoproteins that recognise the phage can be isolated by sequential panning of the library with whole platelets. The phage display system enables the production of large quantities of human Fab fragments.

Results

Specificity of the Isolated Phage

A hundred individual colonies isolated from the phage pool obtained after five rounds of panning were examined for reactivity against platelet proteins using the whole platelet ELISA. These colonies of phage isolated from the fifth round of panning were randomly selected and 10^9 colony forming units (c.f.u.) of phage were added to each well. The anti-phage antibody was employed to detect the binding of this phage to platelets. Of these hundred colonies, just twelve reacted with whole platelets. These were S1, S2, S3, S5, S7, S8, S9, S11, S12, S14, S18 and S20. Fig. 1 shows that the amount of reactivity of the 12 colonies for binding with whole platelets. M13 (helper phage) was used as a negative control and anti-CD41 as a positive control. PBS was the blank. Most of the colonies had absorbance between 0.5 and 0.6, with the highest reactivities against whole platelets being seen around absorbance of 0.6.

Binding of Isolated Phage from Biopanning against Platelet Lysate

The twelve colonies that showed strong reactions against whole platelets were analyzed to determine their degree of binding to the lysate rather than the platelet. Microtitre plates were coated with 100 µl of the platelet membrane lysate at a concentration of 200 µg/ml, and 100 µl of phage was added to each well. Anti-phage antibody was used to detect the degree of binding to the platelet lysate. The results showed that only four colonies from 12 colonies, which reacted with whole platelets, showed substantial reactivity against platelet lysate. S7 showed highest binding to both platelet lysate and whole platelet (Fig. 2).

Reactivity of Phage Isolated from ITP Library against Purified Platelet Glycoprotein IIb/IIIa Complex

The most likely candidate for the antigen recognized by the Fab bearing phages is the platelet glycoprotein GP IIb/IIIa complex. The colonies that showed strong positive reactions against platelet antigens (S2, S7, S8 and S9) were also used to determine their specificity against GP IIb/IIIa. Each well of microtiter plate was coated with 100 µl of purified glycoprotein GP IIb/IIIa at a concentration of 2 µg/ml. Then 100 µl of 10^9 of Fab-bearing phage was added to each well with anti-phage antibody to detect their specificity. The results show that the selected colonies reacted strongly with the GP IIb/IIIa complex compared with the negative control (M13 helper phage), with S7 having the highest reactivity, and S2 the least.

DNA gel agarose

In order to determine that heavy and light chains were present in the phage, DNA of the four phage colonies with stronger reactions to platelet protein were analysed. Again these were the colonies S2, S7, S8 and S9. A plasmid isolation kit was used to isolate DNA from phage grown in Luria broth. The purified DNA from each phage colony was cut with a restriction enzyme and loaded onto an agarose gel before undergoing electrophoresis. The results demonstrated the presence of a DNA band of 5.4 kbp (base pairs) in size. The figure shows the presence of library phage DNA of the correct molecular weight (Fig. 3).
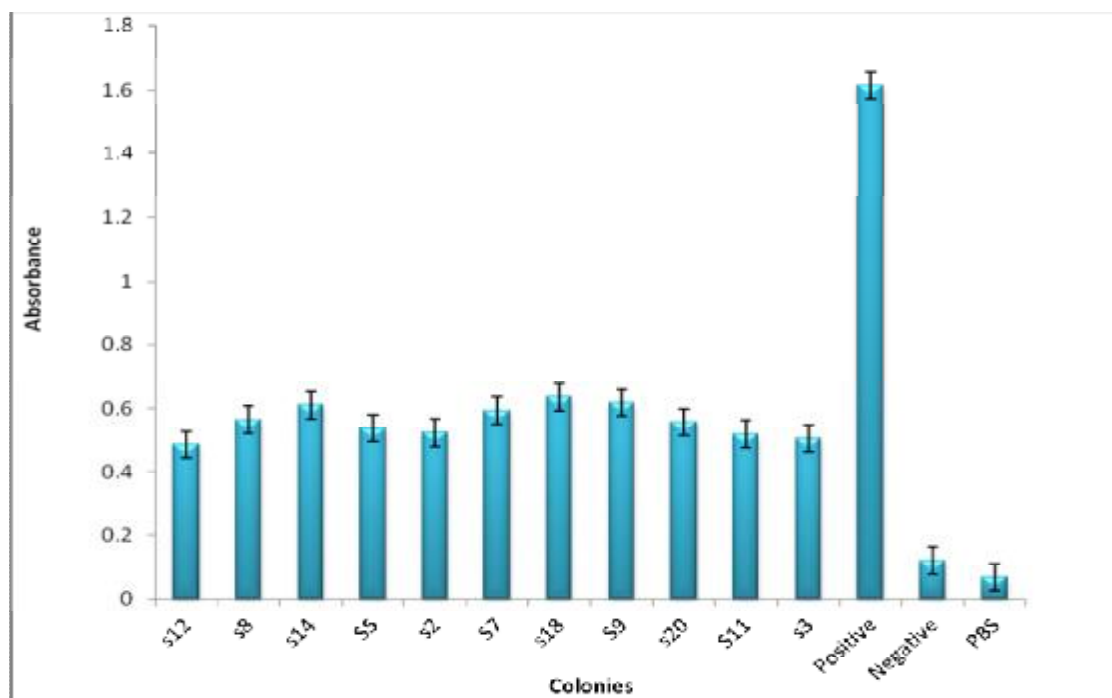

Fig. 1: Whole-platelet binding activity (absorbance) of 12 colonies isolated after five rounds of panning, plus a negative control (M13), a positive control (anti-CD41) and a blank (PBS). In this experiment, 100 µl of whole platelets (10^7/well) was used (section II.7). Data represent the mean (± SE) absorbance of two experiments. Two wells were used for each experiment.
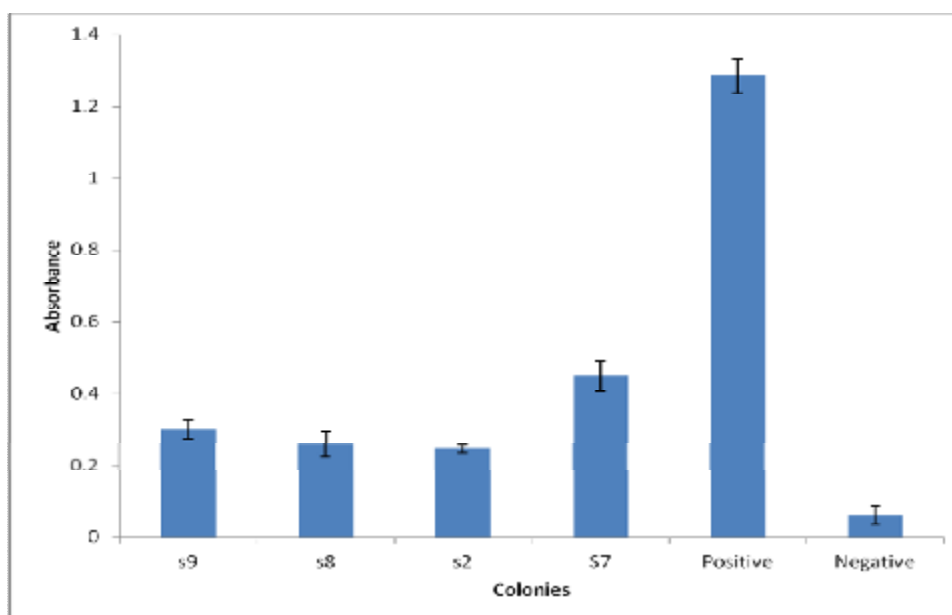
Fig. 2: Reactivity (absorbance) against platelet lysate of phages from four colonies isolated by biopanning. In this experiment, 100 µl of platelet protein (200 µg/ml) was used (section II.5). Anti-CD41 and M13 were used as control positive and negative respectively. Data represent the mean (±SE) absorbance of three experiments. Two wells were used for each experiment.
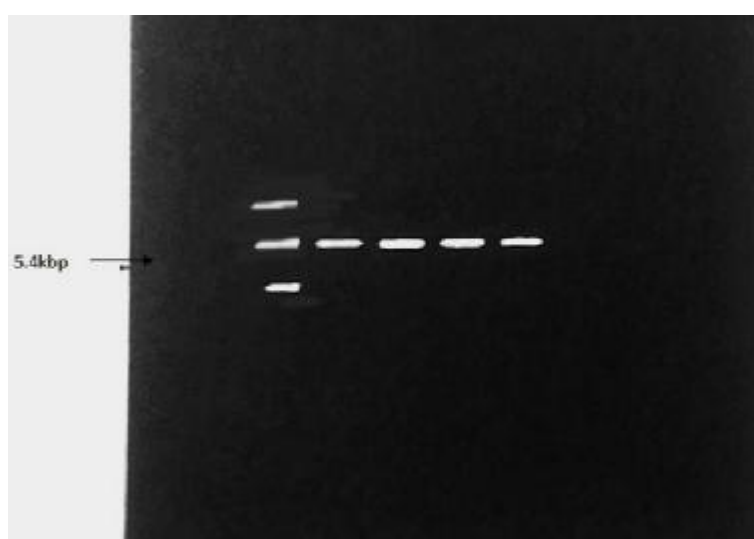

Fig. 3: Purified DNA of the four the phage run on a 1% agarose gel. The four indicated bands (S2, S7, S8, and S9) co-migrated with the 5.4kbp DNA molecular size marker

Discussion

The thrombocytopenia in ITP is characterized by the destruction of platelets in the circulation by antibodies. These antiplatelet antibodies react with various autoepitopes on the glycoprotein complexes, located on the external membrane of platelets, which are the focus of the present study.

ELISA of antiplatelet Antibodies

In the initial stages of the study, a quick and sensitive ELISA assay was developed for analyzing whole washed platelets and frozen platelet lysate from healthy volunteers, in order to identify and quantify the binding of antiplatelet antibodies relevant to the disease ITP. All the glycoprotein subunits on platelets can act as antigens to autoantibodies, but the complexes commonly affected in chronic forms of ITP are the highly immunogenic GP IIb/IIIa and GP Ib/IX complexes (19-22). Around 75% of antiplatelet antibodies in ITP are against antigens on the GP Ib–IX complex, and anti-GP IIb/IIIa autoantibodies occur in approximately 15% of ITP patients (23). It was expected during this study that any antibody reactivity would be directed against one of these types of glycoprotein receptors.

The initial ELISA studies focused on the adhesive protein receptor complex known as GPIIb/IIIa, which is associated with two antigens, namely the CD41 antigen and the CD61 antigen. The GP IIb complex is the CD41 antigen and the GP IIIa complex is the CD61. A reaction was only obtained for anti-CD41, so the subsequent experiments were based on the use of anti-CD41 as control positive. Throughout this discussion, antibodies against the GPIIb complex will be referred to as anti-CD41.

The GP IIb/IIIa complex is of particular significance in ITP as it is also the fibrinogen receptor, which is essential for maintaining normal haemostatic functioning of the body (24-25). Circulating platelet counts decrease through autoantibody-mediated destruction when the glycoprotein receptors are seen as antigens, and disruption of this complex also alters the platelet response to injury. The overall result is the development of bleeding disorders such as purpura and prolonged bleeding time, as seen in ITP.

Having established the presence of glycoprotein antigens in the human platelet samples, various parameters were investigated for optimum performance in the ELISA experiments. These included the choice of blocking buffer. Different kinds of blocking buffers were investigated to find the most appropriate one for use in subsequent ELISAs experiments. The buffer which gave greatest absorbance difference between positive and negative control was 5% dried milk in PBS.

The platelets from the healthy volunteers were prepared in two ways – as a whole platelet preparation or as a lysed platelet preparation. The amount of protein contained in the lysate was determined using a modified Bradford assay (26). Every ml of lysate was found to contain 1400 µg protein. The value of 200 µg protein per ml was used in all subsequent experiments, which it is the same amount of protein of 10^7 whole platelet per well (27). This protein would have included antigens such as CD41 and other glycoproteins, as well as other non-glycoprotein proteins.

ELISA of Antiplatelet Fab Antibody Fragments

ELISA was also used to detect potentially Fab-binding platelet glycoproteins phages. This is because antibody binding to glycoprotein complexes in platelets can be Fab-mediated (28), whereby the Fab-binding part of the glycoprotein binds to the Fab region of the paratope on the antibody.

To detect these platelet Fab-binding glycoproteins, it was necessary to quantify the optimum concentrations of anti-CD41 antibody to use in the ELISA experiments. The optimum concentration was therefore determined using mouse monoclonal anti-CD41 antibody and washed whole platelets. It was found to be 10^{-2} µg/ml.

Then the optimum concentration of whole platelets to use in ELISA experiments was found. Whole platelet preparations in a range of concentrations were tested against anti-CD41 antiplatelet antibody, and a maximum reaction was observed with 10^7 platelets per well. This whole-platelet concentration was used in all subsequent ELISA assays.

The optimum concentration of platelet lysate for detecting anti-CD41 was determined to be 1400 µg/ml that it was used at 200 µg/ml throughout the study.

These optimum concentrations of whole platelets and lysate were used to determine whether whole platelets or lysate were more suitable as a substrate in the binding assays of antibodies and platelet antigens. Whole platelets were expected to adsorb more antibodies against glycoprotein complexes, not least because antiplatelet antibodies can only attach to cation-dependent epitopes on the GP IIIa component if the complex is conformationally intact (23), or because the complex on the platelet surface may undergo a conformational change that causes it to be displayed differently on activated platelets and express new epitopes (29).

In the ELISA analysis of the platelet glycoprotein IIb/IIIa complex, using monoclonal anti-CD41 antibodies, the anti-CD41 antibody reacted most strongly against CD41 antigen in the whole platelet preparation, as expected, compared with the antigen in the lysate preparation. The reaction of the lysed platelets was only 70% of that seen with the whole platelet preparation. There may be several reasons for this. First, the amount of CD41 antigen may have been greater in the whole platelet preparation because there may have been different amounts of protein in the two kinds of samples. Only the protein content in the lysed preparation was determined in this experiment. The protein content of the whole platelet sample may have been different, which meant the two samples could not be directly compared and no assumptions could be made about the amount of CD41 antigen they contained. As a consequence, any differences in the reactivity of the samples in this study had to be treated with caution.

Second, the differences obtained with the different preparations might have arisen because of changes occurring during the preparation of the lysed platelets. Lysis may release non-membrane proteins and expose new antigenic epitopes. The glycoprotein complexes may remains

intact on the surface of the platelet, and some become detached from the platelet membranes and undergo some conformational change. Evidence suggests that some complexes are better recognised by antibody when they are still *in situ* on the surface of the platelet (23). For example, It was showed that antiplatelet antibodies only attach to cation-dependent epitopes on the GP IIIa component when the whole GP IIb/IIIa complex is conformationally intact.

Isolation of Antiplatelet Fab Fragments

Antiplatelet Fab fragments specific for the glycoprotein complex were isolated from a phage library by a procedure known as biopanning, which involves several steps. The biopanning experiments used whole platelets, rather than lysed platelets, largely because the response of autoantibody in ITP is against several glycoproteins or antigens on the surface of the platelet (30).

First, a phage display library was prepared from a Fab-expressing phagemid library of human immunologloglobulin light and heavy antibody chains on the V domain, constructed from the mRNA from the splenic B lymphocytes of a patient with ITP, using cDNA encoding for immunoglobulin mostly IgG. IgG is associated with the chronic form of ITP (15, 31-33), and IgG-specific autoantibodies can be detected in as many as 60% of ITP patients (34) with levels of platelet-binding IgG being shown to increase (33,35)

In this study, PCR-amplified gene segments for the antibody domains which were cloned into PAK100 vectors (18). The desired gene segments were inserted into the phagemid of the *Escherichia coli* and the gene product was displayed on the surface of the bacteriophage. During the capturing step, the phage library was conjugated to the desired target, so that only specific peptides presented by bacteriophage were bound to the target. The bacteria that took up copies of the plasmid became resistant to certain antibiotics, and on exposure to a particular antibiotic, the resistant bacteria survived. These

bacteria were grown, harvested and lysed to isolate the plasmid of interest. Any phages that did not bind to the solid surface are washed away, leaving phages with strong binding affinity. The more times the preparation was washed after each round of panning resulted in the removal of more unbound phage, without significant loss of specifically bound phage.

The library underwent sequential panning with whole platelets from the healthy volunteers, and resulted in the isolation of glycoproteins that recognized the phage specifically. Phage-recognizing platelet antigens that bound the Fab fragments were isolated and the attached phage was recovered and amplified and used to infect more *E. coli*. The more times this was done, the stronger the affinity of the binding peptides to the target.

In order to isolate maximum numbers of potentially anti-platelet antibodies, the biopanning procedure was carried out under different conditions, with either incubation or shaking immediately after adding VCSM helper phage, and with different numbers of washes after each round of biopanning. This study revealed that the number of colonies obtained was maximal with incubation rather than shaking and with five washes after each round of biopanning.

Five rounds of biopanning were carried out. Each round produced higher numbers of enriched antiplatelet antigen-specific Fab-bearing phage. The amount was quantified as colony-forming units (c.f.u.). The first round of panning produced only three clones from a 10^9 c.f.u./ml phage suspension. After five rounds there were 237 colonies. The quantity of starting phage was not significant.

It was expected that these increasingly large numbers colony-forming units would have increased levels of anti- Fab- bearing phage. The specificity of the enriched phage in each round of panning was therefore tested for reactivity against platelet protein. ELISA was used to confirm that the amount of phage potentially bearing anti- Fab increased after each round of panning, reaching a maximum after the five rounds.

The reactivity of a number of colonies after five rounds of panning was investigated next. A hundred colonies were isolated from the phage pool, which randomly were selected. From each of these, 10^9 c.f.u. of the phage were added to a well with anti-phage antibody and examined for reactivity against platelet proteins using the whole-platelet ELISA. Twelve of the hundred colonies showed reactivity against whole platelets. All of the twelve reacting colonies showed a high level of reactivity with whole platelets compared to the negative control M13 phage. The same study showed that 23 out of 40 randomly selection colonies reacted with GPIIb/IIIa that their inhibition studies using murine mAb against various epitopes suggested that binding epitopes of phage antibodies were located within the GPIIb/IIIa complex. They used the phage display approach to isolate Fab antibody fragments recognizing native GPIIb/IIIa.A library from a human donor immunized with Rh⁺ red blood cells was panned with freshly washed platelets (36).

This study used a random selection of phage library colonies to produce Fab antiplatelet antibodies that showed reactivity for certain antigens on the surface of human platelets. This phage library was derived from a patient with the autoimmune disease ITP. ELISA was used to show that the isolated Fab fragments reacted with antigens on the platelet surface. Future studies could be conducted to isolate additional Fab antibody fragments. Further analysis and modification of the fragments isolated in this study, and other Fab fragments isolated in the future, will improve our understanding of the platelet antigen–antibody interaction in the autoimmune disease of ITP.

Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or

falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

Acknowledgements

References

1. George JN, Woolf SH, Raskob GE, Wasser JS, Aledort LM, Ballem PJ, et al. (1996). Idiopathic thrombocytopenic purpura: a practice guideline developed by explicit methods for the American Society of Hematology. *Blood*, 88(1): 3-40.

2. Silverman MA. Idiopathic Thrombocytopenic Purpura: Follow-up 2010.

3. Akwari OEI, Coleman KM, Rosse RE (1987). Splenectomy for primary and recurrent immune thrombocytopenic purpura (ITP). Current criteria for patient selection and results. *Ann Surg*, 206(4): 529-41.

4. Aledort L, Hayward CPM, Chen M-G, Nichol J, Bussel J (2004). Prospective screening of 205 patients with ITP, including diagnosis, serological markers, and the relationship between platelet counts, endogenous thrombopoietin, and circulating ant-ithrombopoietin antibodies. *Amer J Hematol*, 76: 205-13.

5. Block GEE, Zajtchuk R, Splenectomy for idiopathic thrombocytopenic purpura (1966). *Arch Surg*, 92(4): 484-9.

6. George J, Raskob GE, Vesely SK, et al. (2003). Initial management of immune thrombocytopenic purpura in adults: a randomized controlled trial comparing inte-rmittent anti-D with routine care. *Amer J Hematol*, 74: 161-9.

7. Guthrie T, Brannan DP, Prisant LM (1988). Idiopathic thrombocytopenic purpura in the older adult patient. *Amer J Med Sci*, 296: 17-21.

8. Salawu LDM (2001). Immune thrombocytopenic purpura: 11-year experience in Ile-Ife, Nigeria. *African J Med Med Sci*, 30: 99-103.

9. Cines DB, Blanchette VS (2002). Immune thrombocytopenic purpura. *N England J Med*, 346: 995-1008.

10. Kuhne T, Imbach P, Bolton-Maggs PHB (2001). Newly diagnosed idiopathic thrombocytopenic purpura in childhood. *Lancet*, 358: 2122-5.

11. Watts RG (2004). Idiopathic thrombocytopenic purpura: a 10-year natural history study at the childrens hospital of alabama. *Clin Pediatr (Phila)*, 43(8): 691-702.

12. White J. *Platelet structural physiology* 1994.

13. Maton AJH, Charles William McLaughlin, Susan Johnson, Maryanna Quon Warner, David LaHart, Jill D. Wright Human Biology and Health. New Jersey: Englewood Cliffs; 1993.

14. Harrington WJ, Minnich V, Hollingsworth JW, Moore CV (1951). Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. *J Lab Clin Med*, 38(1): 1-10.

15. Shulman NR, Weinrach RS, Libre EP, Andrews HL (1965). The role of the reticuloendothelial system in the pathogenesis of idiopathic thrombocytopenic purpura. *Trans Assoc Am Physicians*, 78: 374-90.

16. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, *et al.* (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, 230(4732): 1350-4.

17. Cheng S, Fockler C, Barnes WM, Higuchi R (1994). Effective amplification of long targets from cloned inserts and human genomic DNA. *Proc Natl Acad Sci U S A*, 91(12): 5695-9.

18. Krebber A, Bornhauser S, Burmester J, Honegger A, Willuda J, Bosshard HR, et al. (1997). Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. *J Immunol Methods*, 201(1): 35-55.

19. Yanabu M, Suzuki M, Soga T, Sone N, Nagata H, Nomura S, et al. (1991). Influences of antiplatelet autoantibodies on platelet function in immune thrombocytopenic purpura. *Eur J Haematol*, 46(2): 101-6.

20. van Leeuwen EF, van der Ven JT, Engelfriet CP, von dem Borne AE (1982). Specificity of autoantibodies in autoimmune thrombocytopenia. *Blood*, 59: 23-6.

21. Woods VL Jr, Oh EH, Mason D, McMillan R (1984). Autoantibodies against the platelet

glycoprotein IIb/IIIa complex in patients with chronic ITP. *Blood*, 63(2): 368-75.

22. Beardsley DS, Spiegel JE, Jacobs MM, Handin RI, (1984). Platelet membrane glycoprotein IIIa contains target antigens that bind anti-platelet antibodies in immune thrombocytopenias. *J Clin Invest*, 74(5): 1701-7.

23. McMillan R (2000). The pathogenesis of chronic immune (idiopathic) thrombocytopenic purpura. *Semin Hematol*, 37(1 Suppl 1): 5-9.

24. Beutler E, Marshal AL, Coller BS, Kipps TJ (1995). Williams' Heamatology. New York: Mc graw Hill; 1995.

25. Stiene-Martin EA, Lotspeinch-Steinger,CA, Koe-pke JA, editor. *Clinical Heamatology*. New York: Lippincott; 1998.

26. Bradford MM (1976). A rapid and sensitive method for the quantisation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72: 248-54.

27. Kelton JG, Steeves K (1983). The amount of platelet-bound albumin parallels the amount of IgG on washed platelets from patients with immune thrombocytopenia. *Blood*, 62(4): 924-7.

28. Stricker RB, Shuman MA (1986). Quinidine purpura: evidence that glycoprotein V is a target platelet antigen. *Blood*, 67(5): 1377-81.

29. Wagner CL, Mascelli MA, Neblock DS, Weisman HF, Coller BS, Jordan RE (1996). Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets. *Blood*, 88(3): 907-14.

30. Smith JW, Hu D, Satterthwait A, Pinz-Sweeney S (1994). Building synthetic antibodies as adhesive ligands for integrins. *J Biol Chem*, 269: 32788-95.

31. Schwartz RS (2007). Immune thrombocytopenic purpura--from agony to agonist. *N Engl J Med*, 357(22): 2299-301.

32. Shulman NR, Marder VJ, Weinrach RS (1965). Similarities between known antiplatelet antibodies and the factor responsible for thrombocytopenia in idiopathic purpura. Physiologic, serologic and isotopic studies. *Ann N Y Acad Sci*, 124(2): 499-542.

33. McMillan R, Longmire RL, Yelenosky R, Donnell RL, Armstrong S (1974). Quantitation of platelet-binding IgG produced in vitro by spleens from patients with idiopathic thrombocytopenic purpura. *N Engl J Med*, 291(16): 812-7.

34. Coopamah MD, Garvey MB, Freedman J, Semple JW (2003). Cellular immune mechanisms in autoimmune thrombocytopenic purpura: An update. *Transfus Med Rev*, 17(1): 69-80.

35. Semple JW (1998). Immunobiology of T helper cells and antigen-presenting cells in autoimmune thrombocytopenic purpura (ITP). *Acta Paediatr Suppl*, 424: 41-5.

36. Escher R, Müller D, Vogel M, Miescher S, Stadler BM, Berchtold P (1998). Recombinant human natural autoantibodies against GPIIb/IIIa inhibit binding of autoantibodies from patients with AITP. *Br J haematol*, 102: 820-8.