Laboratory investigation of immune thrombocytopenia

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Foreword
Many different techniques have been developed for the serological investigation of immune thrombocytopenia. However, because of the unique characteristics of the platelet membrane, precise measurement of pathological antibodies on platelets has been difficult. On the basis of methodology, three types of assays have been used to measure platelet antibodies. Phase I assays measure a platelet-dependent end point after normal platelets are activated by test serum. Because most allo- and autoantibodies do not activate platelets, these assays are not useful, except in the investigation of heparin induced thrombocytopenia. Phase II assays measure either surface or total platelet antibody. These assays are sensitive, but lack specificity for immune thrombocytopenia because they measure both pathological and non-pathological antibodies associated with platelets. Finally, phase III assays measure antibody bound to specific platelet glycoproteins. As these antibodies are targeted at platelet glycoproteins, they are postulated to be pathological. The role of these assays in the diagnosis and classification of immune thrombocytopenia is still evolving.

Introduction
Immune thrombocytopenia can be defined as increased platelet destruction by immune mechanisms in the setting of normal megakaryopoiesis. Idiopathic thrombocytopenic purpura (ITP) is the prototypic immune thrombocytopenic disorder. It is called a primary immune thrombocytopenic disorder because it has no known cause. Immune thrombocytopenia can also be secondary to a number of other disorders such as lymphoproliferative disorders, systemic lupus erythematosus and HIV.

Over 20 years ago, Aster wrote that ITP is a diagnosis of exclusion. This statement remains true today. What also remains true is that a sensitive and specific diagnostic test for ITP, and for most other types of immune thrombocytopenia, remains an elusive goal in platelet immunology.

For many years it has been possible to measure immunoglobulins (as well as many other proteins) associated with platelets. Using sophisticated analytical techniques, it is possible to determine the orientation by which the antibody binds to the platelet (fig 1). However, when the various techniques are analysed, they fail to discriminate between patients with clinically suspected ITP and those with other causes of thrombocytopenia. In this review, we will summarise the application and diagnostic accuracy of techniques used to measure antibodies on platelets.

General definitions
Direct assays measure antibody on platelets, whereas indirect assays measure antibody in the serum. On the basis of the general technique used, as well as the chronological time of introduction, the antiplatelet antibody assays can be divided into three groups. Phase I assays measure platelet activation as the end point. These are biological assays that infer the presence of an antibody-platelet interaction by a platelet dependent end point. Phase II assays measure platelet associated immunoglobulin. Phase III assays, the most recently introduced, directly measure the binding of immunoglobulin to individual platelet glycoproteins.

Phase I assays for antiplatelet antibodies
All phase I assays are indirect assays in which test patient serum is mixed with control platelets. The early phase I assays built on the observations of Harrington and co-workers who noted that plasma from a patient with ITP produced acute and transient thrombocytopenia in the recipient. This simple observation demonstrated that in at least some patients with ITP, the disorder was caused by a circulating plasma factor (subsequently shown to be an immunoglobulin). This plasma factor leads to the destruction of normal platelets.

All phase I assays measure a platelet dependent end point, after mixing patient serum and normal platelets. The end point can be platelet aggregation, platelet release, platelet lysis, or platelet procoagulant formation. The problem with these assays is that, while some antiplatelet antibodies cause platelet activation, many do not. Therefore, phase I assays share a low sensitivity as diagnostic methods for immune thrombocytopenia. With one important exception, phase I assays are no longer in use for the investigation of immune thrombocytopenic disorders.
Figure 1  An illustration of the orientation of antibody binding to platelets in alloimmune, autoimmune and drug dependent thrombocytopenia.

Today, phase I assays are used for the diagnosis of heparin induced thrombocytopenia (HIT). HIT is a disorder characterised by thrombocytopenia and a risk of thromboembolic complications after the administration of heparin. The thromboembolic complications are caused by antibody mediated platelet activation within the circulation. Phase I assays, including the radioiodinated serotonin release assay and a modified platelet aggregation test, are therefore useful for the diagnosis of this condition. The radioiodinated serotonin release assay measures the release of serotonin from labelled normal platelets, incubated with test serum and therapeutic concentrations of heparin. The sensitivity of this assay for HIT is over 90%. The platelet aggregation test, a less sensitive assay, measures aggregation of normal platelets treated in the same way.

Phase II assays for antiplatelet antibodies
Phase II assays were introduced in the early 1970s as techniques to quantify and qualify platelet antibodies. These assays were a step forward from phase I assays because they could measure immunoglobulin bound to the platelets. Phase II assays, however, measure all immunoglobulin bound to platelets, whether pathological autoantibody or non-specific platelet associated immunoglobulin. The results are therefore expressed as platelet associated antibody. Within this group of assays, there are three distinct approaches. These include two step assays, direct binding assays and total platelet associated IgG.

TWO STEP ASSAY
The two step competitive inhibition phase II assays were originally described by McMillan et al and Dixon et al. In McMillan’s Fab anti-F(ab), test, assay, platelets are incubated with a known amount of anti-human IgG. Residual unbound IgG is then quantified by precipitation with ¹²⁵I-labelled Fab fragments. Dixon developed a similar two step assay. In this assay, residual unbound anti-human IgG is quantified by measuring the lysis of IgG coated sheep erythrocytes in the presence of complement.

DIRECT BINDING ASSAYS
Direct binding phase II assays quantify the amount of a labelled ligand bound to the test platelets. The ligand may be monoclonal or polyclonal anti-IgG, or staphylococcal protein A. The IgG is linked to an indicator such as radioactive iodine, horseradish peroxidase or fluorescein. Results of these assays have been expressed variably including ng IgG/10⁶ platelets, to number of IgG molecules per platelet. Typically the assay converts the results using 1:1 binding ratios between primary and secondary antibodies. This may or may not be correct.

TOTAL PLATELET ASSOCIATED IGG (PAIgG) assays for total PAIgG measure both surface bound IgG and IgG from within platelets, mostly from α granules. These methods require detergent lysis of platelet membranes causing release of IgG into solution. The IgG in solution is then quantified by any number of techniques, including immunoprecipitation, nephelometry and radial immunodiffusion. The measurement of total PAIgG is preferred by some laboratories because of the ease of handling platelet specimens. The measurement of total PAIgG results in levels 10 times higher than surface PAIgG, because most PAIgG is in the interior of platelets and not on the surface.

CURRENT CONCEPTS CONCERNING PHASE II ASSAYS FOR PLATELET ANTIBODIES
All three of these assays, two step, direct binding and total PAIgG, have been evaluated in both normal subjects and patients with thrombocytopenia. Although controversies remain, a consensus has been reached about the clinical usefulness of these assays.

Total amount of IgG associated with platelets
Several investigators have measured the amount of surface and total platelet IgG. The average amount of total PAIgG in normal subjects is approximately 20 000 IgG molecules.
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Figure 3 The results of four different assays for PAIgG, representing the different types of PAIgG assays: total PAIgG assays, two stage assays for surface PAIgG, and direct binding assays for surface PAIgG. Normal controls, patients with ITP and non-immune thrombocytopenic patients are presented in relation to the upper normal ranges, 2 SD above the normal mean values. Reprinted with permission.

per platelet. Despite the many different assays and reporting laboratories, there is remarkable agreement on this figure. It has been determined that the majority of PAIgG is found within α granules in the platelet and that less than 1% is on the platelet surface.

IgG on the surface of platelets

The amount of surface PAIgG is less clear. Reported figures vary between 100 and several thousand molecules of IgG per platelet. The difference in amount depends upon the assay used. Direct binding assays, using monoclonal reagents or staphylococcal protein A, typically measure about 200 to 400 surface molecules of IgG per platelet. When a two step assay (fig 2) is used, the average number of IgG molecules per platelet ranges from 2000 to 6000 molecules. It is possible that during platelet preparation, two step assays cause release of IgG from the large internal platelet pool. In this type of assay, any IgG in solution would neutralise the anti-IgG probe, giving falsely raised results. In the direct binding assays, IgG released into solution would not affect the results, as these assays measure only platelet bound IgG.

Direct binding assays, using monoclonal reagents, could result in a small underestimation of the true surface PAIgG. These assays assume a 1:1 binding ratio between the labelled ligand and surface IgG. Studies, however, have shown that binding ratios range from 0.35 to 1.0. At worst, direct binding assays may underestimate the amount of surface PAIgG by threefold.

One study used anti-PL sensitised platelets and measured surface PAIgG using direct binding assays and two step assays in parallel. The two step assay measured 600 000 molecules of IgG per platelet and the direct binding assay measured 20 000 molecules of IgG per platelet. This latter number is more consistent with the known number of copies of glycoprotein Ib/IIa per platelet, which ranges from 40 000 to 80 000 molecules per platelet.

These studies indicate that direct binding assays using monoclonal reagents probably accurately measure the number of IgG molecules on the surface of platelets.

Biological relevance of PAIgG

Each one of the phase II assays discussed earlier has been shown to be sensitive but not specific for immune thrombocytopenia (fig 3). Given the high baseline level of IgG within normal platelets, it is likely that much of the IgG found associated with platelets from patients with immune thrombocytopenia is not pathological. Some investigators have suggested that platelet α granule IgG is obtained by fluid phase endocytosis and that this function is increased when there is a thrombopoietic stimulus. The increased total PAIgG detected in ITP may simply be a reflection of younger platelets under thrombopoietic stimulation. It has also been proposed that increased total PAIgG is a reflection of larger platelets with more α granule IgG. However, we have not previously that there is no relation between platelet volume and total PAIgG. Finally, some investigators have postulated that along with antibody bound via Fab subunits, phase II assays measure immune complexes bound via the Fc receptors. The evidence in this regard is conflicting. We believe that it would be unlikely that immune complexes contribute a significant amount to the level of platelet associated IgG given the low affinity of platelet Fc RI and their low number (about 1000 to 2000 per platelet).

Our laboratory has shown that the amount of albumin bound to platelets parallels the amount of PAIgG in patients with immune thrombocytopenia. Other investigators have shown that some autoantibodies cause platelet activation. We have speculated that some IgG binds to platelets in a specific Fab dependent fashion, causing activation or damage to the platelet. This in turn leads to a change in the platelet membrane and increased non-specific binding of plasma proteins and IgG to the platelet surface. This change in the platelet surface may account for some of the non-
specific binding of IgG in ITP. Thus, although increased PAIgG is a sensitive indicator for ITP, it is likely that much of the total PAIgG detected represents non-pathological IgG.  

SUMMARY
In summary, there is a consistent and more importantly predictable variability in the amount of IgG associated with platelets as measured by each general type of phase II assay: (a) total PAIgG, which measures internal and external IgG, gives the highest amount of PAIgG; (b) direct binding assays, using monoclusal reagents, give the lowest amount of IgG on the surface; and (c) two step assays give intermediate results. When the same test platelet samples are measured in parallel using the three different types of assay, each provides the same sensitivity and specificity when compared with the control range as determined by that assay. What has also been clear is that while most patients with immune thrombocytopenia have raised PAIgG, irrespective of the assay used, many patients with non-immune thrombocytopenia (for example, leukaemia and aplastic anaemia) also have raised levels of PAIgG. This latter observation might suggest to some investigators that these assays give erroneous results. This conclusion is inaccurate. There is remarkable agreement among the assays and among many different investigators. What remains uncertain is the mechanism of the elevated PAIgG in the non-immune thrombocytopenic patient and how the body handles these IgG sensitised platelets.

Phase III assays for antiplatelet antibodies

Given the low specificity of phase II (PAIgG) assays, new techniques have been developed to try to overcome this problem. Phase III assays share a common approach: they measure the binding of antibody to a specific platelet glycoprotein. This strategy was first suggested by the work of van Leeuwen and associates who showed that antibody eluates from ITP patients’ platelets bound to normal platelets but not to platelets of patients with Glanzmann’s thrombasthenia. Because thrombasthenic platelets are deficient in platelet glycoprotein IIb/IIIa, this observation suggested that patients with ITP had antibody directed against these platelet glycoproteins. Since 1984, many phase III assays have been developed. Their advantage over phase II assays is that they measure platelet specific antibodies rather than non-specific platelet/antibody interactions (PAIgG). The assays can be divided into three: immunoblotting, specific antigen capture assays and immunoprecipitation.

IMMUNOBLOTTING

The immunoblot assay is an indirect assay. Here, washed normal platelets are solubilised in SDS. The platelet proteins are then separated by polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose. The nitrocellulose strips are then cut and incubated with test serum. Localisation of bound antibody can be with radiolabelled or enzyme linked anti-human IgG. The molecular weight of the glycoproteins bound by human IgG determines their identity.

Beardsley et al were the first to demonstrate antibody binding to platelet glycoproteins with the immunoblot assay. The antibody was derived from the serum of patients with post-transfusion purpura (PTP) and ITP, and the target platelet glycoprotein was shown to be glycoprotein IIIa. Since that report, investigators have noted autoantibodies directed against glycoprotein IIb/IIIa and other target proteins in 20–90% of patients with ITP. Furthermore, serum from patients with ITP often binds to multiple platelet antigen targets, suggesting multiple autoantibody specificities in each patient. The immunoblot assay has also been used to investigate alloantibodies, autoantibodies, and drug dependent antibodies.

There are several inherent problems with the immunoblot assay. For example, when normal serum samples are studied using this technique, up to 85% have been shown to produce reactions similar to those seen in autoimmune disease. This suggests that at least some of the findings in ITP may be non-specific. Also, platelet glycoproteins in the immunoblot are subject to powerful denaturing influences. As a result, not all antibodies react with transfblotted glycoproteins. When the monoclonal antibody specific immobilisation of platelet antigens assay (MAIPA; see later) and immunoblot assay were compared in 30 patients with chronic ITP, only three autoantibodies were identified by immunoblot while 17 were identified by MAIPA. In addition, our laboratory has noted that alloantigens PL and Bak can be detected by immunoblotting, but the alloantigens Zav (Br) and Gav cannot. Given these problems, the role of the immunoblot assay in the investigation of immune thrombocytopenia is limited.

ANTIGEN CAPTURE ASSAYS

The antigen capture assays use monoclonal antibodies to capture specific platelet glycoproteins. An anti-human globulin probe, either monoclonal or polyclonal, is used to determine whether antibodies are bound to the glycoprotein of interest. These assays permit precise identification of antibody bound to a defined platelet glycoprotein. The antigen capture assays are technically simple and usually do not require radioactive isotopes. There are, however, drawbacks to this group of assays. For example, some auto- or alloantibodies may share antigenic epitopes with monoclonal antibodies, resulting in steric hindrance and false negative results. To overcome this problem, a cocktail of monoclonal antibodies, with different specificities, should be used in each assay. Monoclonal antibodies, however, are not always available or may be costly. An additional drawback is that the design of this type of assay does not permit identification of novel platelet glycoprotein/antibody interactions.

There are three types of antigen capture assay (fig 4): the microtitre well assay, the MAIPA assay and the immunobead assay.
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Microtitre plate assay

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MAIPA assay

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Immunobead assay

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<tr>
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Figure 4 An illustration of antigen capture assays.

Microtitre well assay

The microtitre well assay is an indirect assay first described by Woods et al. Each well of a microtitre plate is coated with a monoclonal antibody directed against a platelet glycoprotein of interest. The wells are then incubated with platelet lysate prepared from normal platelets. After a wash step, test plasma is added to each well. After a second wash, a mono- or polyclonal anti-human globulin probe, which is radiolabelled or enzyme linked, is added to detect antibody bound to the platelet glycoproteins.

The microtitre well assay has been used in the study of patients with auto- and alloimmune antibodies. Sensitivity for the detection of platelet autoantibodies in ITP ranges from 3% to 30%, but is generally less than 10%. Serum samples from both normal subjects and patients with non-immune thrombocytopenia have been negative in these studies.

Experience with the microtitre well assay has shown the signal to noise ratio to be low. This is postulated to be because of non-specific binding of IgG from patient plasma to the plastic surface of the wells. In addition, although this assay seems to be quite specific for ITP, it has a low sensitivity. Solubilisation of platelet antigens before sensitising with antibody disrupts antigenic epitopes recognised by the autoantibody or monoclonal antibody, making them inaccessible for binding.

Monoclonal antibody specific immobilisation of platelet antigens

The MAIPA can be used as a direct or an indirect assay, simply by using test platelets or washed normal platelets sensitised with patient serum. Similarly to the microtitre well assay, the MAIPA uses a monoclonal antibody to capture the platelet glycoprotein of interest.

The advantage of the MAIPA over the microtitre well assay is the preservation of antigenic epitopes in the platelet glycoprotein. This is accomplished by sensitising the platelet with murine anti-glycoprotein monoclonal antibody and human autoantibody, before solubilisation. The platelets are then lysed in non-ionic detergent. Microtitre plates, coated with goat anti-mouse antibodies, are then used to capture the glycoprotein–murine monoclonal antibody complex. A final antibody, either mono- or polyclonal anti-human globulin, is used to determine whether antibodies are bound to the platelet glycoprotein of interest.

The MAIPA has other advantages over the microtitre well assay. For example, by avoiding contact between patient plasma and the plastic microtitre well surface, there is less non-specific binding of patient IgG to the plastic surface and less background noise. However, there is an inherent disadvantage to the MAIPA. Some normal subjects have been found to have anti-mouse antibodies in their serum. These antibodies can react with the monoclonal antibodies used in the MAIPA, giving false positive results. This problem is avoidable if platelets are serially incubated with test serum and then with the murine anti-glycoprotein monoclonal antibody.

The MAIPA has been useful in the investigation of both auto- and alloantibodies. The existence of private platelet alloantigenic systems, such as the Sr and Va systems, was first suggested by the findings of serological cross-matches between maternal serum and paternal platelets using the MAIPA assay. The MAIPA has also been used in preliminary studies in the assessment of ITP. The indirect MAIPA can detect autoantibodies in the serum of approximately 30% to 50% of patients with ITP. The direct MAIPA has been reported to detect autoantibodies directed against platelet glycoprotein IIb/IIIa or Ib/IX in 75% of patients with ITP. The sensitivity of these preliminary studies may be an overestimation as some used pre-selected ITP serum samples known to have platelet reactive antibodies by prior phase II (PAIgG) screening assays. When unscreened ITP serum samples are used the sensitivity is lower. Few normal subjects or non-immune thrombocytopenic controls have been investigated with the MAIPA. Finally, the MAIPA has been used to study the specificity of autoantibodies in patients with immune thrombocytopenia and antiphospholipid antibodies. In these studies, adosorption experiments revealed no cross-reactivity between antiphospholipid and anti-glycoprotein IIb/IIIa autoantibodies. Although
antiphospholipid antibodies are present in the serum of 20 to 40% of patients with ITP, their contribution to the pathogenesis of immune thrombocytopenia remains unclear.13

Immunobead assay

The immunobead assay and the MAIPA are very similar.48 The immunobead assay can be used as a direct or an indirect assay. Antigenic epitopes on the platelet glycoproteins are preserved in both assays by sensitising the platelets before solubilisation. The immunobead and the MAIPA differ in one feature: in the immunobead procedure, the platelet glycoprotein is captured onto a solid phase by the murine monoclonal antibody directed against the glycoprotein of interest, rather than by a second antibody directed against the murine monoclonal antibody.49 A radio-labelled antihuman globulin probe, either mono- or polyclonal, is used to detect antibody bound to the platelet glycoprotein.

The immunobead assay has been used most frequently in the investigation of autoantibodies in patients with ITP. In the MAIPA, the test platelets are first sensitised with a polyclonal antihuman IgG antibody and then exposed to radiolabelled antibody. The immunobead assay has also been used prospectively to measure autoantibodies in patients with ITP undergoing therapy.50 51 These preliminary studies demonstrate that the level of platelet autoantibodies parallel the patients' response to immunosuppressive therapy.

The role of platelet capture assays in the investigation of immune thrombocytopenia is still unfolding. A platelet serology workshop, comparing the MAIPA to a phase II (PAIgG) assay, has shown the MAIPA to be a more sensitive and reproducible assay in the study of alloantibodies.65 Workshops studying ITP serum produced findings that have been less encouraging than single institution studies.64 65 No single assay can identify all autoantibodies in the serum of patients with ITP, a finding that may be a reflection of the monoclonal antibodies used by different laboratories.

Antigen capture assays have been used to make some novel observations about ITP. For example, it has been shown that the specificity of platelet associated versus plasma autoantibodies in ITP often differs.66 Plasma antibodies can be directed against cytosolic domains of platelet glycoproteins, which may be exposed when platelets are destroyed.67 Platelet associated autoantibodies are directed against topographic epitopes that depend on the three dimensional structure of platelet glycoproteins.68 The biological importance of these findings is unclear.

Immunoprecipitation

Our laboratory has experience using this procedure. Immunoprecipitation can be a direct or indirect assay. In the direct assay, test platelets are surface radiolabelled (125I) or labelled with biotin, before lysis. In the indirect assay, normal platelets are first labelled and then sensitised with human serum prior to lysis. Antibody-glycoprotein complexes are then immunoprecipitated from the solution using staphylococcal protein A bound to beads. The complexes are then subjected to SDS polyacrylamide gel electrophoresis. The gels are dried and autoradiographed, and the glycoproteins identified by molecular weight.

Immunoprecipitation has been used for the investigation of both allo- and autoantibodies. While our laboratory has found the technique to be very useful in the identification of novel platelet alloantigen systems, few studies have used immunoprecipitation in the investigation of ITP.69 Tomiyama et al69 studied six patients with ITP using direct immunoprecipitation. Four of the six patients had autoantibody directed against platelet glycoprotein Iib/IIIa. In the same series, three of the patients with anti-Iib/IIIa were followed prospectively before and after splenectomy. Similarly to those patients studied with immunobead assays, the presence of anti-Iib/IIIa was found to correlate with the patients' response to therapy.69 70 71

The immunoprecipitation assay offers several advantages over other phase III assays. Antigenic targets of allo- and autoantibodies are preserved by sensitising platelets before lysis. This should improve the sensitivity of the assay over the immunoblot and the microtitre well assay. As monoclonal antibodies are not required for immunoprecipitation, steric hindrance between monoclonal antibodies and allo- or autoantibodies is not a consideration. Furthermore, some alloantigens are epitopes on proteins for which no monoclonal antibodies are yet available.72 Finally, immunoprecipitation is a superior assay for the detection of novel platelet antibody-antigen systems.73 The assay, however, is technically difficult and in the past has required the use of radioactive isotopes. To date, it has not been applied extensively to the study of ITP.

SUMMARY

The phase III assays may overcome the problem of specificity of platelet antibody that plagued the phase II assays. Phase III assays measure antibody directed against specific platelet glycoproteins. For this reason, investigators infer that the antibody being measured with these techniques is pathological antiplatelet antibody. Within the group of assays, some techniques have advantages over others. For example, the MAIPA, immunobead and immunoprecipitation assays preserve antigenic epitopes by sensitising platelets before lysis. Because of the use of monoclonal antibodies, the MAIPA and immunobead assays permit precise identification of the antigen targeted by allo- or autoantibody. In preliminary studies the MAIPA and immunobead assays have been shown to be useful in the study of ITP, identi-
Laboratory investigation of immune thrombocytopenia

I. Introduction

Laboratory investigation of immune thrombocytopenia through thrombocytopenia of selected data has raised the importance of raised immune thrombocytopenia. Thus, the detection of immune thrombocytopenia still advances, however, with the development of new assay methods.

II. Methods

Our approach to this problem involves the detection of autoantibodies in the sera of patients with immune thrombocytopenia. The specificity of these autoantibodies is assessed by their ability to bind normal platelets in vitro.

III. Results

Autoantibodies were found in the sera of patients with immune thrombocytopenia. These autoantibodies were shown to be specific for the platelet membrane glycoprotein GPIb.

IV. Conclusion

These results suggest that autoantibodies may play a role in the pathogenesis of immune thrombocytopenia. Further studies are needed to clarify the role of these antibodies in the diagnosis of immune thrombocytopenic purpura.

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