DETECTION OF ANTIPLATELET ANTIBODIES IN PATIENTS WITH IDIOPATHIC THROMBOCYTOPENIC PURPURA (ITP) BY IMMUNOFLUORESCENCE

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SYNOPSIS

An immunofluorescence test (IFT) using platelet suspensions was used to detect the presence of serum anti-platelet antibodies (APA) in the sera of Malaysian patients with idiopathic thrombocytopenic purpura. Of the 28 patients tested, 19 (or 68%) had detectable APA with percentage platelet fluorescence ranging from 34% - 80% (mean 51% ± 10). Normal sera gave fluorescence values of 6 - 15% (mean 9% ± 5). Sera from patients with SLE, thyrotoxicosis and dengue haemorrhagic fever gave mean values of 29%, 8% and 9% respectively. Additionally, no apparent correlation was observed between percentage platelet fluorescence and the severity of thrombocytopenia. The importance and significance of these findings are discussed.

INTRODUCTION

Idiopathic thrombocytopenia purpura (ITP) is believed to be an auto-immune disease where the destruction of platelets in a majority of patients with this disorder is mediated by an auto-reactive anti-platelet antibody (1, 2). Following the early studies of Harrington (3) ample evidence has been obtained for an immune mechanism of platelet destruction involving antiplatelet antibodies (APA) (4, 5). Since a diagnosis of ITP is reached largely by a process of *exclusion,* the development of methods to detect antiplatelet antibody (APA) **in vitro** would be a valuable aid in the assessment of patients with suspected ITP. In relation to this numerous methods hav been developed to detect the presence of APA in the serum of ITP patients or to demonstrate APA bound to the patients' own platelets. Due to various factors, however, many of these methods are complex and tedious to perform and have given results which are equivocal, unpredictable and often unrepeatable (1).

In consideration of the above, we set out to assess various methods to detect serum APA in Malaysian patients with ITP. The immunofluorescence test (IFT), originally developed by Borne et al (6), was chosen because of its relative simplicity and convenience. Additionally, although the incidence of APA is well documented in Caucasian ITP cases (7, 8), very little is known of the occurrence of APA in Asian patients with ITP.

MATERIALS AND METHODS

Patients

A total of 28 patients with ITP were selected for the study. A diagnosis of ITP was reached by a process of exclusion of all other causes of thrombocytopenia e.g. drugs, on both clinical and laboratory grounds. A bone marrow biopsy or aspirate was also obtained for examination. Other patients with confirmed diagnoses of systemic lupus erythematosus (SLE), thyrotoxicosis (Graves' disease) and dengue haemorrhagic fever were also selected as specificity controls. Normal, healthy controls were obtained from the student population at the Faculty of Medicine, University of Malaya or from blood bank donors.

Serum specimens

Blood was collected by venepuncture and allowed to clot at room temperature. Sera was then inactivated at 56°C for 30 minutes and, if not immediately tested, dispensed into plastic tubes in aliquots of 0.3 ml and stored at −70°C until use.

Isolation of platelets from blood

Blood from a normal, healthy donor was collected into EDTA and centrifuged at 1000 rpm (MSE bench centrifuge) to separate platelet-rich plasma from packed red blood cells. Platelets were then isolated from the platelet-rich plasma by centrifugation at 2000 rpm for 15 minutes and then washed three times in 0.3% EDTA-PBS (pH 6.5). After counting, platelets were resuspended in EDTA-PBS at a concentration of 5×10^5/ul.

Immunofluorescence test (IFT) to detect APA

The methods used in the IFT test were modifications from methods published previously (7, 8). 0.025 ml of platelet suspension was mixed with 0.025 ml of patient's serum and incubated for 60 minutes at room temperature. Platelets were then washed three times with 0.3% EDTA-PBS (pH 6.5). Following complete removal of the supernatant after the final wash, 0.05 ml of FITC-conjugated anti-human IgG (1 in 10 dilution; Wellcome Laboratories, Beckenham, England) was added and the mixture incubated for 30 minutes at 4°C. Platelets were then washed again three times and finally resuspended in one drop of EDTA-PBS, transferred to a glass microscope slide and covered with a coverslip. The slide was then examined with a Leitz Ortholux II fluorescent microscope (incident illumination) and the percentage of platelets fluoresceing estimated.

RESULTS

Of the 28 ITP sera tested, 19 (or 68%) were positive for APA (Table I) with percentage platelet fluorescence ranging from 34% to 80% (Fig. 1) and a mean value of 51% ± 10 (Table I). Normal, control sera gave 6 - 15% fluorescence with a mean of 9% ± 5 (Table I, Fig. 1). Sera from patients with SLE, thyrotoxicosis and dengue haemorrhagic fever were also tested as specificity controls and gave mean platelet fluorescence values of 29%, 8% and 9% respectively (Table I). It should be noted that only 1 out of 6 SLE cases had significant levels of APA (50% fluorescence) (Table I). It was also found that there was no apparent correlation between the degree of thrombocytopenia and the percentage of fluoresceing platelets (Fig. 2).

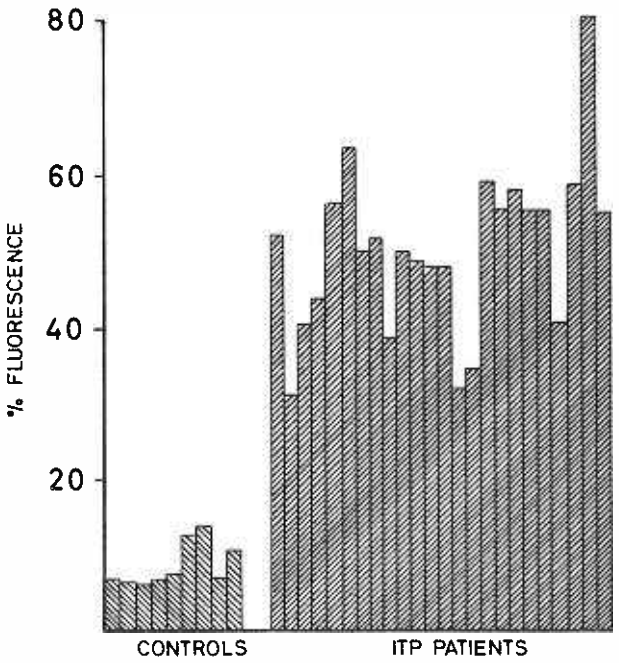

Figure 1. Detection of APA in ITP patients.

TABLE I Detection of APA by Immunofluorescence

Diagnosis	Mean platelet count	No. tested	No. positive	% platelet fluorescence	Mean % platelet fluorescence	% Positive
ITP	15,000 (3,000 - 70,000)	28	19	34 - 60	51 ± 10	68
SLE		6	1	20 - 50	29 ± 10	17
Thyrotoxicosis		4	0	10	8 ± 4	0
Dengue	30,000	4	0	10	9 ± 2	0
Control	150,000-400,000	10	0	6 - 15	9 ± 5	0

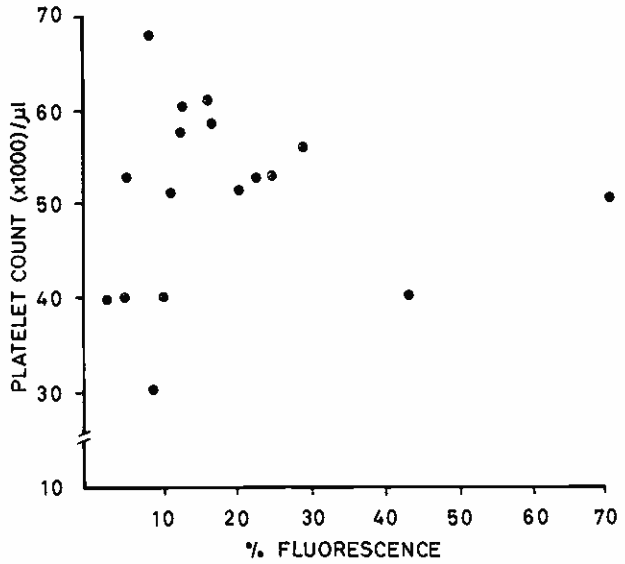

Figure 2. Relationship between percentage platelet fluorescence and thrombocytopenia.

DISCUSSION

The detection of APA **in vitro** has proven to be difficult for several reasons. Platelets have an inherent tendency to aggregate and to adhere to various surfaces (9). Also the APA, although originally shown to be an IgG immunoglobulin (4), appears to stick non-specifically to the platelet surface (10), antigenic specificity for the immunoglobulin has not been demonstrated and the APA appears to be non complement-fixing (2). Despite these problems many methods have been developed to detect APA (1). Many of these methods, however, are tedious and have suffered from a lack of reproducibility and no one method is in general use. Of the tests to detect serum APA (by binding to the surface of normal platelets and subsequent 'immune injury' or other effects on the platelets), the platelet complement fixation test (11) and the ^{51}Cr-platelet lysis test (12) are both subject to variation and rely on the complement-fixing ability of the APA and will thus not detect APA which is non-complement fixing. Both the platelet factor 3 and serotonin release test (13, 14) are based on biological assay and chemical measurement and are too cumbersome for clinical application. On the other hand, other tests have been designed to detect APA bound on the patients' own platelets. The antiglobulin consumption assay (15), for example, has gained acceptance as a sensitive, reliable, and reproducible test. It is, however, tedious to perform due to the multiple procedures involved and, due to its complexity, requires considerable technical skills. Its main disadvantage, however, lies in the fact that it requires considerable amounts of blood from patients who are already severely thrombocytopenic. Indirect immunofluorescence (6) has been used to detect platelet-bound APA and has been shown to be a simple, reliable test but still disadvantageous in its requirement for patient's blood.

In consideration of the above, we decided to use immunofluorescence as a means of detecting serum APA in Malaysian patients with ITP. Results obtained indicate that it is a reliable convenient and simple to perform test with good sensitivity. The inherent problem of platelet aggregation was overcome to a large extent by the use of EDTA-PBS as a washing solution at a pH of 6.5, in contrast to a pH of 7.4 used previously (8). Using this test it was found that approximately 68% of patients with ITP had detectable APA in their sera with values ranging from 34 - 80% platelet fluorescence. The background fluorescence observed with normal sera was around 9%. To test for specificity, sera from patients with other diseases were also tested. One of the SLE patients tested showed some binding to platelets (around 50% fluorescence) but little or no binding activity was detected in the sera of patients with thyrotoxicosis, another autoimmune disease, and in the sera of patients with dengue haemorrhagic fever where thrombocytopenia is a marked feature of the disease. In relation to studies done in Caucasian subjects, Borne et al (8) used a platelet suspension immunofluorescence test and found that 45 - 69% of ITP patients had APA. Veenhoven et al (7) demonstrated by IFT that serum APA was found in 59% of patients with active disease but in only 27% of those who had attained remission. These results agree quite well with those obtained in the present study.

The data obtained also showed some variability in the percentage platelet fluorescence ranging from 34% to 80% (Fig. 1). Hirschman and Shulman (14) have suggested that this variability may be a result of differing affinities of the APA for the platelet surface. For example, in patients with a high affinity antibody, most or all of the APA would be adherent to the patients' own platelets with only negligible amounts remaining in the serum. This may account for some low percentages of fluoresceing platelets seen in patients who are severely thrombocytopenic (Fig. 2). It also serves to highlight another important finding in the course of the present study namely that there appears to be no apparent correlation between percentage platelet fluorescence and the severity of thrombocytopenia (Fig. 2). This lack of correlation between the presence of APA and disease severity has also been noted by others (15, 16) and thus implies that a low percentage or absence of fluoresceing platelets does not indicate the absence of APA or the degree of thrombocytopenia.

At present, testing for APA in ITP must be considered of limited value in clinical practice: the diagnosis of ITP cannot be established nor can other disorders be excluded by their detection. However, the result of this and other investigations have provided techniques for the direct detection of APA which will be of value in the assessment of ITP cases. Of more practical importance is the fact that the APA can be characterized as IgG or IgM. As IgG antibodies are able to cross the placenta this characterization may be of importance in the management at delivery in pregnant women with ITP.

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