

Detection of Platelet Antibody Using Streptavidin-Biotinylated β -Galactosidase Complex[†]

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= Abstract = A new immunocytochemical method for the detection of platelet antibody was developed using streptavidin-biotinylated β -galactosidase complex. The method was evaluated in terms of its sensitivity with samples from ITP patients and platelet antibody positive sample tested by the immunofluorescence method.

All 34 positive sera for platelet antibody by the immunofluorescence method were also positive with the streptavidin-biotin method. Eight (50.0%) of 16 ITP patient sera were positive by the streptavidin-biotin method, however, only five (31.3%) of 16 sera were positive by immunofluorescence method.

The average platelet antibody titer obtained by the streptavidin-biotin method was 7.5 times higher than that by the immunofluorescence method. The platelets bound on glass slides could be stored at 4-8°C for up to 3 weeks.

In summary, the new immunocytochemical method using streptavidin-biotinylated β -galactosidase could be used for the detection of platelet antibody with higher sensitivity than the immunofluorescence method.

Key words: *Platelet antibody, Immunocytochemistry, Streptavidin-biotinylated β -galactosidase complex*

INTRODUCTION

It is well known that the platelet antibody is frequently detected in patients with idiopathic thrombocytopenic purpura (ITP), thrombocytopenia in newborn infants, post-transfusion purpura, systemic lupus erythematosus (SLE) and in those patients who persistently show thrombocytopenia on repeated platelet transfusion (Moore 1982).

Although many aspects of platelet antibody problems still remain to be solved, an improved technique for antibody detection is needed not only for proper diagnosis and treatment but also to investigate the pathogenesis and progress of platelet disorders (Moore *et al.* 1984).

Various methods such as complement fixation (Hegde *et al.* 1977), immunofluorescence (IF) (von dem Borne *et al.* 1978, 1980; Cha *et al.*

1983; van Leeuwen *et al.* 1981; Boisvert and MacPherson 1983), antiglobulin consumption (Sims and Boswell 1983; Orsini *et al.* 1985), nephelometry (Pawha *et al.* 1983), radial immunodiffusion (McMillan 1981), radioimmunoassay (RIA) (Cheung *et al.* 1983 & 1983; Faig and Karpatkin 1982; Hirschman and Schulman 1973; Hymes *et al.* 1979), enzyme immunoassay (ELISA) (Lynch *et al.* 1985; Winiarski and Holm 1983; Nel *et al.* 1983; Forster and Schmidt 1983; Hegde *et al.* 1981 and 1985; Tamerius *et al.* 1983; Yesus *et al.* 1984; Schiffer *et al.* 1983; Gudino and Miller 1981; Kahane *et al.* 1981) and immunocytochemistry (Leporrier *et al.* 1979; Tate *et al.* 1977; Schmidt *et al.* 1980) have been developed for the detection of platelet antibody. However, the sensitivity and specificity of these available methods seem to be relatively low compared with red cell antibodies.

Among them the immunofluorescence and enzyme immunoassay methods are most widely used currently in Korea; however, the former is characte-

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rized by rather low sensitivity and specificity, while the latter method using peroxidase or alkaline phosphatase is complex and suffers relatively low reproducibility.

The streptavidin-biotinylated β -galactosidase complex is a recently introduced substance having high potential for immunocytochemistry, enzyme immunoassay and protein blotting. This method seems to have higher sensitivity and stability compared with the peroxidase-antiperoxidase method widely used in immunocytochemistry.

The authors made an investigation to develop an immunocytochemical method for platelet antibody detection with this streptavidin-biotinylated β -galactosidase complex.

MATERIALS AND METHODS

1. Patients

Sixty-nine serum samples sent to Seoul National University Hospital laboratory for the detection of platelet antibody were studied. They included 26 cases of ITP, 7 multiply transfused patients, 2 Evans' syndrome, 1 SLE and 33 follow-up unavailable patients. Platelets and sera for control study were obtained from 10 healthy adult volunteers who were not taking any medications for reasonably long period. All sera were stored at -70°C until tested.

2. Reagents

Anticoagulant: 5% $\text{Na}_2\text{-EDTA}$

Buffers: Phosphate buffered saline(PBS), pH 7.4 and $\text{Na}_2\text{-EDTA-PBS}$, pH 6.8-7.0

Biotinylated species-specific anti-human immunoglobulin, whole antibody(Amersham, England)

Streptavidin-biotinylated β -galactosidase complex(Amersham, England)

5-bromo-4-chloro-3-indolyl β -D-galactopyranoside(BCIG, Sigma, U.S.A.)

Working BCIG solution: 0.05 ml of solution A were added to 2.3 ml of solution B. The mixed solution can be stored for at least 2 months at -18°C , or can be stored for 1 month at $4-8^{\circ}\text{C}$ with sodium azide.

Solution A: 10 mg of BCIG were dissolved in 0.5 ml of dimethyl formamide.

Solution B: 7 ml of PBS containing 1mM MgCl_2 were added to 0.5 ml of 50 mM potassium ferricyanide and to 0.5 ml of 50 mM potassium ferrocyanide.

3. Methods

A) Principle

The complex method is illustrated as shown in

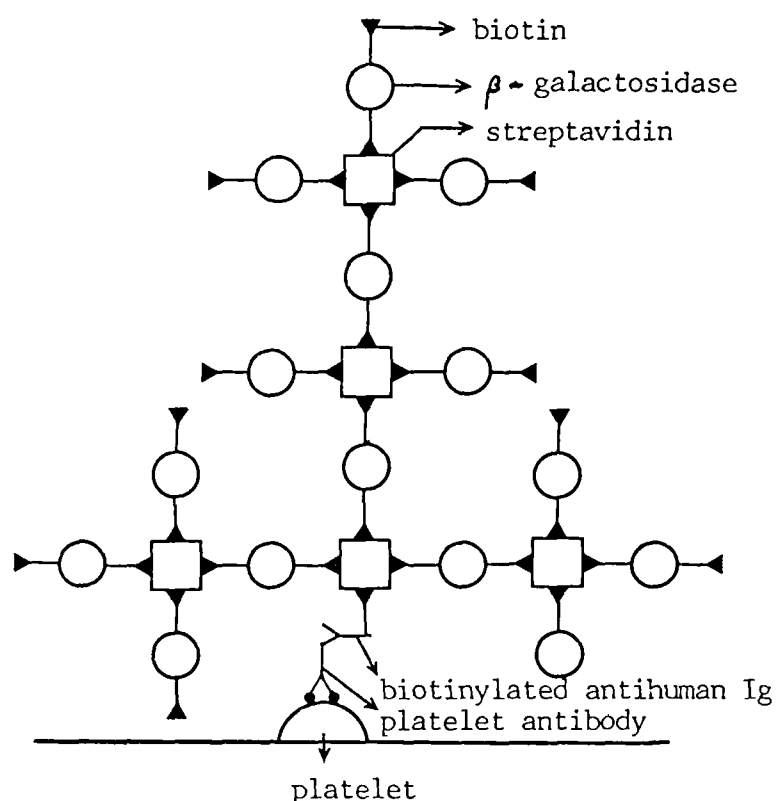


Fig. 1. Illustration of streptavidin-biotin method for the detection of platelet antibody.

Fig. 1. Normal platelet bound slides are prepared and serum samples suspected to have platelet antibody are applied on it. Biotinylated antihuman immunoglobulin is then overlaid following the application of streptavidin-biotinylated β -galactosidase complex. Finally, BCIG is applied as a substrate for cytochemistry. To evaluate the reaction, the slides are read under a light microscope with a 1000-fold magnification. The positive reaction is light blue against an unstained background (Fig. 2). The detailed procedure for the technique is described in Table 1.

B) Preparation of platelet bound glass slides

Platelet-rich plasma (PRP) was harvested from $\text{Na}_2\text{-EDTA}$ anticoagulated group O whole blood by centrifugation at 800 g for 15 minutes. The volume of whole blood was 10 ml including 1 ml of 5% $\text{Na}_2\text{-EDTA}$. After recentrifugation at 2800 g for 10 minutes, the platelets were washed 5 times with EDTA-PBS and resuspended. The platelet count was then adjusted to $4-10 \times 10^8/\text{ml}$. The glass slides were coated with one drop of platelet suspension. After 10 minutes, the platelets had settled and were firmly attached to the glass area. The slides were then drained and unbound platelet suspension removed. Drying at room temperature for one day was satisfactory. After drying, the platelet bound slides were stored at $4-8^{\circ}\text{C}$ and -70°C .

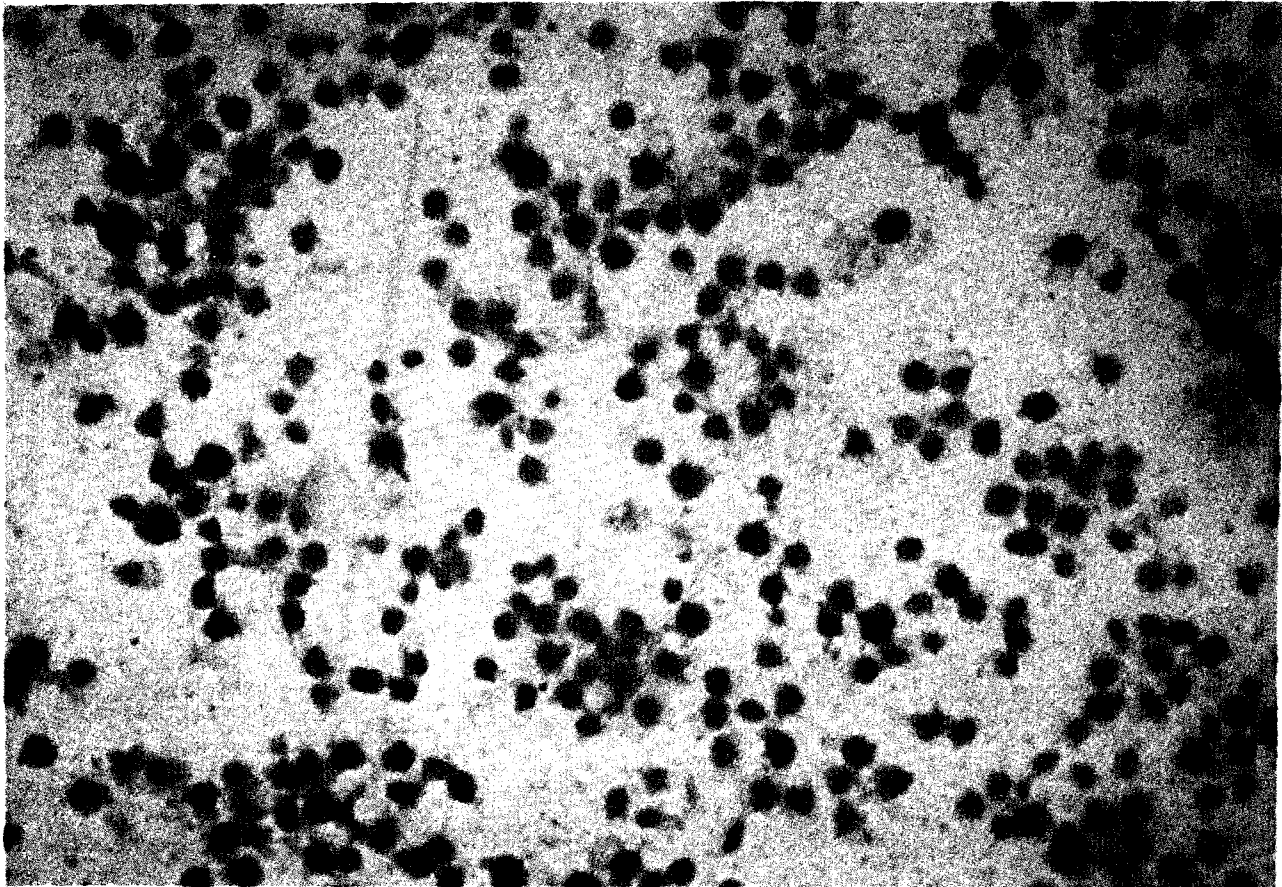


Fig. 2. Positive results with streptavidin-biotin method.

Table 1. Procedure for streptavidin-biotin method

1. Prepare the platelet bound slides.
2. Add patient's serum (1:4 diluted) and incubate at room temperature for 1 hour.
3. Wash by immersing slides in PBS buffer without agitation. Repeat washing three times with PBS buffer solution.
4. Add biotinylated antihuman immunoglobulin and incubate at 37°C for 1 hour.
5. Wash three times with PBS buffer.
6. Add streptavidin-biotinylated β -galactosidase complex and incubate at 37°C for 30 minutes.
7. Wash three times with PBS buffer.
8. Add BCIG solution and incubate at 37°C for 90 minutes.
9. Wash three times with PBS buffer.
10. Examine with light microscope.

RESULTS

1. Comparison of streptavidin-biotin method and immunofluorescence method

The sensitivity of the streptavidin-biotin method was compared in 69 patients with that of the IF

Table 2. Diagnoses of the patients with platelet antibody detected by both streptavidin-biotin method and immunofluorescence method

Diagnoses	Number
Idiopathic thrombocytopenic purpura	15
Multiple transfusion	7
Evans' syndrome	1
Unknown	11
Total	34

method which has been used in our laboratory. With the IF method, 34 patients of the total showed positive reaction while 35 negative results. All of the 34 positive sera by the IF method were also positive by the streptavidin-biotin method. 15 of the 34 were ITP, 7 were multiply transfused patients, 1 was Evans' syndrome, and 11 were follow-up unavailable patients (Table 2). Twelve of 35 negative sera by the IF showed positive reaction by the streptavidin-biotin method. Five of the 12 were ITP (Table 3). In 16 cases of ITP, 8 showed positive results by the streptavidin-biotin method, and its sensitivity was 50.0%. Since only 5 of the 16

Table 3. Comparison of immunofluorescence method and streptavidin-biotin method

Methods		Immunofluorescence method		Total
		Positive	Negative	
Streptavidin-biotin method	Positive	34	12	46
	Negative	0	23	23
Total		34	35	69

Table 4. The sensitivities of streptavidin-biotin method and immunofluorescence method for platelet antibody in ITP patients

Method	Number of sample	Number of positive case(%)
Streptavidin-biotin	16	8(50.0%)
Immunofluorescence	16	5(31.3%)

Table 5. The platelet antibody titers detected by streptavidin-biotin method and immunofluorescence method

Sample number	Streptavidin-biotin	Immunofluorescence
1	1 : 32	1 : 4
2	1 : 16	1 : 2
3	1 : 16	1 : 2
4	1 : 32	1 : 2
5	1 : 4	1 : 1
6	1 : 16	1 : 4
7	1 : 32	1 : 2
8	1 : 8	1 : 4
9	1 : 16	1 : 2
10	1 : 16	1 : 2
Average titer	1 : 18.8	1 : 2.5

showed positive results by the IF method, its sensitivity was 31.3% (Table 4). All normal controls showed negative results.

2. Test results of serially diluted sera

Serially diluted sera of 10 platelet antibody positive patients were tested by both methods. The average platelet antibody titer obtained by the streptavidin-biotin method(1:18.8) was 7.5 times higher than that by the IF method(1:2.5) (Table 5).

3. Use of stored platelets and stored platelet bound slides

Using platelet suspension stored at -70°C for 1

month, the platelets were aggregated or destroyed. Using platelet bound glass slides stored at -70°C, the reactions were weakened. Storage of platelet bound slides at 4°C for up to 3 weeks did not influence the results.

DISCUSSION

Since the 1950s or early 1960s, researchers have made efforts to detect platelet antibody and a variety of methods have been developed (Harmon and Miller 1981) for that purpose.

Immunofluorescence is one of the simple detection techniques in clinical laboratories. The sensitivity of the indirect method in ITP has been reported about 40-44% (van Leeuwen *et al.* 1981; von dem Borne *et al.* 1980). In Korea, the reported detection rate was only 12%(Cha *et al.* 1983). IF also has several disadvantages. Specialized microscope is required for the examination of IF preparations, preparations stained by means of FITC-labeled antibody are impermanent, and fresh platelets are needed (Taylor 1978). Therefore, there have been many studies to solve these problems and develop a more sensitive, simple, and specific method.

Recently, a sensitive immunocytochemical method was developed and employed in the studies of platelet antibody and leukocyte antigen. In general, it was reported that peroxidase-antiperoxidase (PAP) complex is as much as 1000 times more sensitive than IF (Bourne 1983). But in the

study of platelet antibody, PAP method was proved to be two to eight times more sensitive than IF (Schmidt *et al.* 1980).

There has been no report that avidin-biotin complex was applied to study of platelet antibody. It was reportedly applied only to the studies of terminal deoxynucleotidyl transferase and leukocyte antigen, and its reported sensitivity was the same as PAP (Lanham *et al.* 1985) or 2-4 times sensitive than PAP (Henke *et al.* 1984). In this study, streptavidin-biotinylated β -galactosidase complex, using streptavidin instead of avidin, was used and the result was 7.5 times more sensitive than IF. Therefore, it can be said that the similar result was obtained from PAP.

ELISA method is considered a good method in a sense that its results can be read objectively. But its results are not more sensitive than that of the IF method (Miller and Harmon 1983). Furthermore, it has the defect that it cannot recognize nonspecific binding of IgG or conjugate to a test tube occupying 7% of total nonspecific binding (Gudino and Miller 1981).

Separation of pure platelets is essential to ELISA and RIA. In cases of direct assay, at least 28-40 ml of blood must be collected for less than 30,000/ml platelet count. Since pure platelets are not essential to immunocytochemistry and the buffy coat can be used, the required blood amount is less than that required by ELISA or RIA.

Using indirect methods which detect platelet antibody in the sera, 10-12 sera can be tested from one donor platelet suspension by the IF method. About 150 sera can be tested from one donor platelet suspension by immunocytochemistry of the streptavidin-biotin method.

The use of stored platelet bound glass slides at 4-8°C for 3 weeks results in similar reactions from fresh preparations. It is thus a time saving method.

Streptavidin is a protein with the same biotin binding property as avidin without the disadvantages of avidin, which is highly positively charged at neutral pH and binds nonspecifically to lectin.

In the immunocytochemistry, peroxidase is the most widely used enzyme, but endogenous peroxidase activity, the need of fresh substrates and carcinogenic activity of substrates are major problems.

Streptavidin-biotin method appears to have the same sensitivity and specificity as the PAP method. No carcinogenic activity has been demonstrated so far for the BCIG nor for the final products of the reaction, and the solutions of the enzyme-conju-

gated antibodies and substrate are stable for several months (Bondi *et al.* 1982).

Streptavidin-biotin method is a very sensitive and convenient method of immunocytochemistry.

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= 국문초록 =

Streptavidin-Biotinylated β -Galactosidase 복합체를 이용한 혈소판항체 검사법에 관한 연구

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Streptavidin-biotinylated β -galactosidase 복합체를 이용하여 혈소판항체 검사법을 개발하고 이를 기존 면역형광법과 비교한 결과 다음과 같은 결과를 얻었다.

1. 면역형광법으로 양성인 34명의 혈청을 streptavidin-biotin법으로 검사한 결과 모두 양성이었다.
2. ITP 환자 16명의 혈청을 검사한 결과 8명이 양성이어서 민감도는 50.0%였다. 이는 면역형광법의 민감도 31.3%보다 높았다.
3. Streptavidin-biotin법과 면역형광법으로 10개 검체의 혈소판항체 역가를 측정할 때 streptavidin-biotin법으로 측정할 때 역가가 7.5배 높았다.
4. 혈소판이 부착된 슬라이드는 3주 동안 냉장보관하여 사용하여도 신선한 표본과 차이가 없었다.

이상의 결과로 streptavidin-biotinylated β -galactosidase 복합체를 사용하여 효소면역세포 화학법으로 혈소판항체를 증명할 수 있음을 확인하고 이 방법이 현재 가장 널리 쓰이고 있는 면역형광법보다 우수함을 확인하였다.