

# Platelet antibodies in immune thrombocytopenic purpura and onyalaï

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## Summary

A prospective study was undertaken to assess the nature, incidence and natural history of platelet antibodies in patients with immune thrombocytopenic purpura (ITP) and patients with onyalaï, using an immunofluorescent technique. Twelve patients under 14 years old and 11 patients 14 - 75 years old with ITP, and 24 patients with onyalaï were studied.

Alternate younger patients were treated with corticosteroids. Ten of the 12 children with ITP had IgG platelet antibodies in their serum, which disappeared as the platelet count recovered. Steroid therapy did not change the course of the disease or the antibody response.

Of the 24 patients with onyalaï, 23 had IgG antibodies and 18 had IgM antibodies, which were still present after 14 days and unrelated to the rise in platelet count. Steroid therapy did not affect the platelet count or the antibody titre.

The difference in immune response of ITP and onyalaï points to a difference in aetiology. The clinical presence of IgM antibodies in onyalaï fits the hypothesis that a toxin, possibly acting as a hapten, is responsible for this form of thrombocytopenia.

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In the serological investigation of immune thrombocytopenic purpura (ITP) it is important to demonstrate the presence of antibodies against platelets. *In vitro* studies for this purpose have included agglutination, complement fixation, inhibition of complement fixation, antiglobulin consumption and <sup>125</sup>I-labelled antiglobulin measurements in serum. Dixon *et al.*,<sup>1</sup> using a quantitative antiglobulin consumption technique, were able to demonstrate increased IgG on the platelet surface, but the method is too complex for routine diagnostic use.

Serological techniques have long been in use but have led to considerable problems.<sup>2</sup> Immunofluorescence tests on platelets have in the past been hampered by nonspecific fluorescence caused by non-immunological adsorbance of immunoglobulins to the platelet surface. Recently it has been shown that fixation with paraformaldehyde (PFA) will prevent this without altering cell surface antigens.<sup>3</sup> PFA fixation permits the stabilization of

antigens at any desired moment during the process of capping and it inhibits non-immune binding of immunoglobulins to platelets, granulocytes and lymphocytes. An alternative possibility is that PFA inactivates IgG Fc receptors on such cells, but does not hinder specific binding of antibodies.<sup>3</sup>

We report on the use of this immunofluorescent technique in the study of patients with ITP and onyalaï. The sera from these patients were studied using pooled platelets, granulocytes and lymphocytes from normal blood donors. F(ab)<sub>2</sub>-rabbit-antihuman (-RH) IgG-antiglobulin reagent was supplied by the Department of Immunohaematology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Centre, Amsterdam. In principle this reagent is prepared as antibodies against human IgG in rabbits. The serum was adsorbed with purified proteins until it was specific for human  $\gamma$ -chains in a passive haemagglutination test. It was then submitted to partial enzymatic digestion<sup>4,5</sup> and the resulting F(ab)<sub>2</sub> fragments were finally conjugated to fluorescein isothiocyanate (FITZ). The background fluorescence obtained with these globulin fragments is significantly lower than with the native antibody molecule and is independent of the presence or absence of Fc receptor-bearing cells.

## Patients and methods

### Patients

The sera from 47 patients with ITP or onyalaï were investigated for platelet, granulocyte and lymphocyte antibodies. Twelve patients, 7 females and 5 males under 14 years old, and 11 patients, 9 female and 2 males between 14 and 75 years old, had a clinical diagnosis of ITP. Two female patients with subacute lupus erythematosus (SLE) and thrombocytopenia were also studied. The sera from the 24 patients with onyalaï were taken on the 1st and the 14th day of the acute illness. The last-named sera were supplied by Dr Amadhila from Oshakati Hospital, SWA, and transported as air freight in a frozen state.

The diagnosis of ITP in the adults was based on a bleeding disorder in the presence of thrombocytopenia, absence of preceding infection or drug ingestion, an increased number of megakaryocytes in the bone marrow aspirate and trephine biopsy specimens, and negative screening tests for other auto-immune disorders.

### Controls

Twenty-six patients with thrombocytopenia and hypoplastic or aplastic anaemia or pancytopenia associated with various conditions were studied in a similar manner to the above 47 patients, while another 26 patients with acute or chronic leukaemia, lymphoma or metastatic carcinoma, 41 patients with minor blood transfusion reactions and 10 normal volunteers from the medical and medical technology staff were screened for the presence of platelet, neutrophil and lymphocyte antibodies.

### Methods

The indirect platelet immunofluorescent test as described by von dem Borne *et al.*<sup>6</sup> was used. The sera were examined with

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commercial FITZ anti- $\gamma$ -globulin (Behring Institute) and sheep-antihuman IgG-, IgM- and IgA-FITZ-antiglobulin preparations (Wellcome Laboratories). The sera of the patients with ITP and onyalai were studied with the F(ab)<sub>2</sub>-RH IgG-antiglobulin reagent. The indirect immunofluorescent test for the determination of granulocyte antibodies and of lymphocyte antibodies<sup>7</sup> was carried out with FITZ-Ig-antihuman globulin reagent.

For each batch of tests the Western Province Blood Transfusion Centre kindly supplied us with blood from 5 normal donors of blood group O. From these specimens a platelet pellet was separated from the pooled platelet-rich plasma and washed three times in EDTA-PBS (phosphate-buffered saline).<sup>7</sup> The lymphocytes were separated on a Ficoll-Hypaque gradient according to the method of Boyüm.<sup>8</sup> In this gradient the pellet contains the granulocytes with some red blood cells. The contaminating red cells are lysed for 5 minutes at 4°C with 2 ml 0,9% NH<sub>4</sub>Cl-PBS containing 1% EDTA.<sup>7</sup>

The platelet, lymphocyte and granulocyte pellets from the 5 donors were pooled separately, washed three times in EDTA-PBS and fixed by resuspension in 1% PFA for 5 minutes at room temperature. After a further three washings, the PFA-fixed platelet, lymphocyte and granulocyte suspensions were incubated with the complement-inactivated test sera for 1 hour at room temperature. After incubation the cell suspensions were again washed three times and incubated with the appropriate fluorescein-labelled antiglobulin preparation at optimal dilution for 30 minutes at room temperature. The cells were then washed twice, resuspended and examined as wet preparations under an immunofluorescence microscope. For a negative control test, pooled group O cell suspensions were put up against complement-inactivated AB serum. The positive control test was a platelet suspension from a person of blood group A put up against serum containing anti-A. A Wild-Leitz SM-LUX microscope with epi-illumination using the standard filter set in the blue excitation range with excitation filters BP 450/490 + FT 510 and barrier filter LP 520 was used. The percentage of fluorescent cells was judged by the scoring of at least 200 cells, using alternately blue incident and phase contrast light. Reactions were scored as positive only when more than 20% of cells were fluorescent and if there was definite evidence of 'ring' fluorescence (Fig. 1).

Platelets were counted on Coulter Model S Plus apparatus at the Department of Haematology, Tygerberg Hospital, and on a Coulter Model S at Oshakati Hospital.

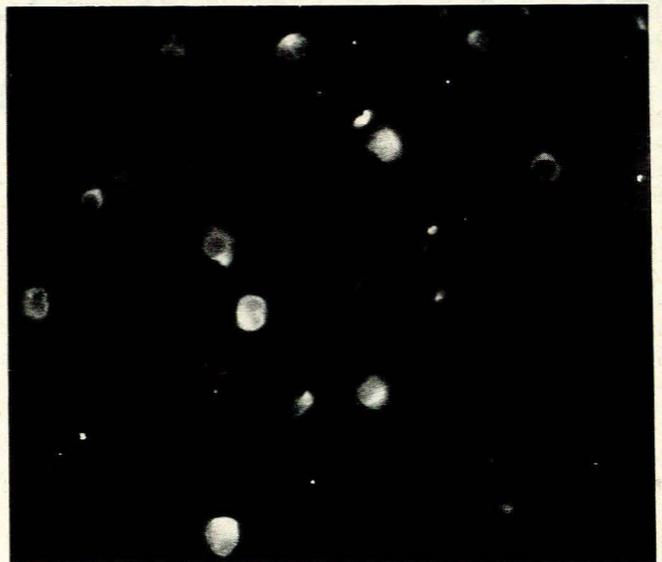


Fig. 1. Membrane fluorescence of human platelets. Incubation was performed with F(ab)<sub>2</sub>-RH IgG-antiglobulin reagent after fixation with PFA, demonstrating the presence of IgG platelet antibodies (x 1250).

## Results

### ITP patients under 14 years

Platelet antibodies were found in the sera of 10 of the 12 ITP patients under 14 years old. The antibody reacted most strongly with the FITZ-anti- $\gamma$ -globulin preparation and the F(ab)<sub>2</sub>-RH IgG-antiglobulin reagent, indicating that it was an IgG antibody. In 2 patients there was evidence of weak IgM antibodies and 1 patient had IgA antibodies. The investigations were repeated at intervals during the course of treatment (Table I). Statistically there was a significant association with the Ig antibody levels, which tapered off as the platelet count improved ( $P < 0,01$  using the Student *t* test, and  $P < 0,02$  using non-parametric tests). Alternate patients were treated with corticosteroids but the treatment did not influence the course of the disease or the antibody response (Fig. 2). Platelet antibodies could not be demonstrated in the sera of 2 patients, and it appears that they had a more resistant or chronic form of ITP. One patient had evidence of neutrophil IgG antibodies and in 1

TABLE I. ANTIBODY RESPONSE AND PLATELET COUNTS IN 12 PATIENTS UNDER 14 YEARS WITH ITP

Patient	Sex	Age (yrs)	Immunofluorescence (%)									Steroids
			1st Day				14th - 25th Day					
			Platelets (x10 <sup>9</sup> /l)	Ig	F(ab) <sub>2</sub> -RH-IgG	IgM	Platelets (x10 <sup>9</sup> /l)	Ig	F(ab) <sub>2</sub> -RH-IgG	IgM		
1	F	8	12	70	—	—	167	0	—	—	No	
2	M	5	20	75	80	—	458	30	60	—	No	
3	F	10	16	75	70	—	153	40	30	—	Yes	
4	F	10/12	12	20	40	30	106	—	—	—	No	
5	M	4	16	80	80	—	250	50	30	—	Yes	
6	F	1-2/12	48	50	50	—	73	30	60	—	Yes	
7	F	1-5/12	46	60	50	—	310	—	40	—	No	
8	M	10	30	80	30	—	76	80	40	—	No	
9	F	1	15	60	50	—	91	30	25	—	Yes	
10	M	10	14	—	—	—	ND	—	—	—	No	
11	F	3	20	60	—	30	ND	—	—	—	No	
12	M	8	—	—	—	—	—	—	—	—	Yes	

ND = not done.

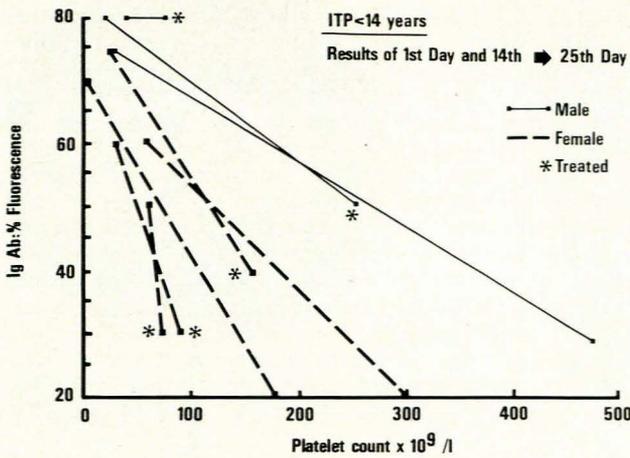


Fig. 2. Association between Ig antibodies, percentage immunofluorescence and platelet counts in ITP patients under 14 years old.

patient lymphocyte IgG antibodies were demonstrated. These findings could be related to a nonspecific antibody reacting with platelets and granulocytes or lymphocytes.

**Patients with onyalai**

The sera from the 24 patients with onyalai reacted with Ig and IgG fluorescein antiglobulin reagents and in 18 patients there was evidence of IgM antibodies (Table II). Nine patients still had evidence of IgM antibodies after 14 days and, in contrast with ITP patients, there was no statistically significant association with the platelet counts. In 2 patients lymphocyte Ig antibodies were demonstrated. Alternate patients were treated with corticosteroids but this did not influence the course of the disease or the antibody response (Fig. 3).

**ITP in older patients**

Platelet antibodies were demonstrated in 7 of the 11 older

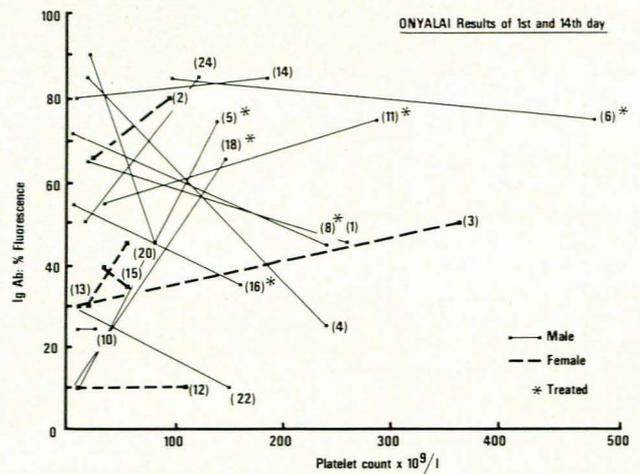


Fig. 3. Association between Ig antibodies, percentage immunofluorescence and platelet counts in patients with onyalai.

patients with ITP. These were mostly IgG antibodies and in 2 patients there was evidence of weak IgM antibodies only. These patients had frequently been on treatment before being referred to the Haematology Clinic at Tygerberg Hospital and the disease had been present for a variable length of time. They all received large doses of prednisolone; depending on the response, splenectomy was performed. One patient with SLE and thrombocytopenia but with no platelet antibodies had a resistant type of thrombocytopenia and responded partially to vincristine but poorly to splenectomy.

**Controls**

In 2 of the 26 thrombocytopenic patients and in 8 of the 26 patients with malignant haematological disorders antibodies of a nonspecific nature, reacting also with granulocytes and lymphocytes, were found. These antibodies could be related to sensitization after blood transfusion. Nonspecific platelet

**TABLE II. ANTIBODY RESPONSE AND PLATELET COUNTS IN 24 PATIENTS WITH ONYALAI**

Patient	Sex	Age (yrs)	Immunofluorescence (%)									Steroids
			1st Day				14th Day					
			Platelets (x10 <sup>9</sup> /l)	Ig	F (ab) <sub>2</sub> -RH-IgG	IgM	Platelets (x10 <sup>9</sup> /l)	Ig	F(ab) <sub>2</sub> -RH-IgG	IgM		
1	M	10	22	65	30	45	264	45	25	50	No	
2	F	21	20	65	90	78	97	80	75	45	No	
3	F	60	8	30	—	—	361	50	35	—	No	
4	M	19	23	85	80	—	240	25	55	40	No	
5	M	16	10	—	—	—	142	75	45	25	Yes	
6	M	7	96	85	80	75	492	75	25	40	Yes	
7	M	7	2	50	60	70	—	40	35	—	Yes	
8	M	49	5	72	—	—	240	45	25	45	Yes	
9	F	5	44	65	80	65	234	—	—	—	No	
10	M	10	11	25	—	40	23	25	—	—	No	
11	M	9	39	55	45	—	287	75	75	—	Yes	
12	F	18	10	—	—	—	109	—	—	—	No	
13	F	22	55	45	30	—	20	30	30	—	No	
14	M	6	9	80	80	28	185	85	70	25	No	
15	F	12	35	38	30	25	51	35	—	—	No	
16	F	18	5	55	30	25	161	35	25	—	Yes	
17	M	10	10	25	—	—	—	—	—	—	No	
18	M	12	6	—	—	25	147	65	30	25	Yes	
19	F	32	—	33	—	25	—	50	25	25	yes	
20	M	6	25	90	60	45	83	45	35	20	No	
21	M	11	13	—	—	—	97	35	45	—	Yes	
22	M	2	5	30	45	20	149	—	25	—	No	
23	F	5	4	65	80	65	234	—	—	—	No	
24	M	11	17	50	65	30	123	85	65	20	No	

antibodies were also found in 20 of the 41 patients with minor allergic reactions to blood transfusion. The sera from the 10 control subjects were uniformly negative for platelet, granulocyte and lymphocyte antibodies.

## Discussion

A prospective study was started by Dr P. B. Hesseling of the Department of Paediatrics during April 1979 in order to: (i) demonstrate platelet antibodies in the serum of patients younger than 14 years with ITP and patients with onyalaï; (ii) assess the nature of the antibodies present; (iii) investigate the course of the disease in relation to the level of antibody; (iv) assess the influence of steroid therapy.

### ITP in patients under 14 years and onyalaï

In the sera of the young patients with ITP and those with onyalaï, clear-cut positive reactions were found with random donor platelets of blood group O. In ITP the specificity of the antibodies for platelets in most cases was confirmed by negative results with granulocytes and lymphocytes. There was a statistically significant association between platelet antibody levels and the platelet count, the antibody apparently being mostly IgG; in 2 patients IgM antibodies were demonstrated and in 1 patient IgA antibodies. The recovery in platelet count was associated with a disappearance of the antibodies. In 2 patients antibodies against granulocytes or lymphocytes were found. In the patients with onyalaï the antibodies appeared to be IgG and IgM and the antibody levels did not fall with the patient's recovery and the higher platelet count on the 14th day. The difference in the immune response between patients under 14 years with ITP and patients with onyalaï was statistically significant ( $P < 0,05$  using the Student *t* test, and  $P < 0,02$  with non-parametric tests). Lymphocyte antibodies were found in the sera of 2 patients. Prednisolone did not influence the course of the disease or the antibody response in these patients (Figs 1 and 2), as previously reported by Metz *et al.*<sup>9</sup>.

The difference in the immune response in ITP and in onyalaï points to a different aetiology for the two conditions. This is consistent with the fact that in ITP there is a recognized immunological mechanism for the thrombocytopenia.<sup>2</sup> Gildenhuis<sup>10</sup> formulated the hypothesis that a toxin from a specific fungus which contaminates the staple food, *amuhango*, of Blacks in SWA during storage is responsible for onyalaï.

### IgM antibodies in onyalaï serum

The clinical picture and the prevalence of IgM platelet antibodies in the serum from patients with onyalaï fit the hypothesis that a toxin, acting perhaps as a hapten, is responsible for the thrombocytopenia. In some cases of thrombocytopenia due to hydrochlorothiazide and in one associated with rifampicin an IgM antibody was documented.<sup>11</sup> There is now convincing evidence that the drug acts as a hapten. *In vitro*, the interaction between drug, antibody and platelet leads to platelet injury and rapid sequestration; *in vivo*, it is manifested by various serological phenomena, including platelet agglutination, lysis and complement fixation. Neither the antibody nor the drug is active alone. IgM auto-antibodies also appear to be commoner in auto-immune thrombocytopenia.<sup>12</sup>

### Platelet antibodies after blood transfusion

It is well recognized that the clinical picture of patients with minor or 'allergic' types of blood transfusion reactions is related to platelet and/or white cell antibodies.<sup>13</sup> It is significant that in 20 of the 41 patients nonspecific antibodies were found reacting with platelets, granulocytes and/or lymphocytes.

### ITP in older patients and SLE

Ten of the 11 older patients with ITP and the 2 patients with SLE and thrombocytopenia all received large doses of prednisolone as therapy and underwent splenectomy. Platelet

antibodies were demonstrated in 7 of the test sera during the initial examination but not later. An association with the recovery of platelet counts could therefore not be demonstrated. Karpatkin and Siskind<sup>14</sup> have suggested that the spleen may be a primary source of antiplatelet antibody, as evidenced by the disappearance of this antibody after splenectomy. Von Bostel *et al.*<sup>15</sup> believe that by means of immunofluorescence microphotometry a detailed serological investigation is possible in auto-immune thrombocytopenia, comparable to that with the antiglobulin test in auto-immune haemolytic anaemia. Such an investigation is complete only if the patient's platelets are included in the investigations, since auto-antibodies are quite frequently demonstrable only on the platelets. It is perhaps significant that in the 1 patient with SLE and with poor response to prednisolone therapy and splenectomy, platelet antibodies were not demonstrable.

## Conclusions

The laboratory detection of platelet antibodies in auto- and allo-immune thrombocytopenia has become more satisfactory. Inadequate numbers of platelets in patients with ITP at presentation may preclude the demonstration of auto-antibodies.<sup>16</sup> It is perhaps also significant that in the 1 patient in whose serum no antibodies could be demonstrated a more resistant 'adult' type of ITP seemed to develop. The test could be of value in distinguishing the acute from the chronic type of ITP in children. It is clear from this study that in thrombocytopenic patients with ITP or onyalaï there is a greater chance of platelet antibodies being present than in patients with isolated thrombocytopenia due to a hypoplastic or aplastic anaemia with a depressed bone marrow.<sup>15</sup> This shows that thrombocytopenia is not in itself responsible for the stronger fluorescence, which was also specific for a particular antiglobulin reagent.

In our series, although the patient's own platelets were not included in the tests (this was frequently precluded by the severity of the thrombocytopenia), immunofluorescence detection and quantitation of platelet antibodies in ITP and onyalaï has become an important parameter in the diagnosis and management of the illness.

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