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EDTA-Dependent Pseudothrombocytopenia – Clinical and Serological Study of 217 Cases

EDTA-zależna pseudotrombocytopenia – badania kliniczne i serologiczne 217 przypadków

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Abstract

Background. Pseudothrombocytopenia (PTCP) involves a spuriously low platelet count associated with *in vitro* platelet agglutination due to the presence of antiplatelet antibodies reacting with platelets in blood drawn into ethylenediaminetetraacetic acid (EDTA).
Objectives. Clinical and serological analysis as well as follow-up of 217 PTCP cases diagnosed in Poland in 1989–2007.
Material and Methods. EDTA-dependent antiplatelet antibodies were tested by the platelet immunofluorescence test (PIFT) using FITC-labeled antiglobulin conjugates against human IgG and IgM. PIFT results were evaluated by flow cytometry and microscopy.
Results. Of the group of 217 PTCP cases, 39% were healthy subjects and 61% were patients with mostly vascular, infectious, autoimmune, liver, and metabolic diseases. EDTA-dependent antibodies were detected in 97% of the cases, IgG antibodies in 19%, IgM in 34%, and IgG+IgM in 44%. IgG antibodies were more frequent in the healthy subjects than in the patients ($p < 0.01$) and IgM more frequent in patients ($p < 0.025$). Follow-up was performed in 88 PTCP patients (mean follow-up time: 4 years, range: 1–18 years). New diseases were determined in 31% of the cases (5 initially healthy, 22 previously ill subjects). The frequencies of the new diseases (autoimmune, metabolic, neoplastic, vascular, infectious) did not differ from those in the Polish population ($p > 0.05$). EDTA-dependent antibodies were still present in 81% of the PTCP subjects. Pseudoleukocytosis was determined in 34% of the healthy persons with PTCP.
Conclusions. These observations indicate that 1) PTCP does not predispose to the occurrence of the above-mentioned diseases; it is therefore likely that EDTA-dependent antibodies have no clinical significance, 2) it is extremely important to differentiate pseudothrombocytopenia and thrombocytopenia to avoid unnecessary treatment, 3) in EDTA-dependent PTCP cases, platelet count should be performed in blood collected in an anticoagulant other than EDTA or immediately following blood collection in a Bürker chamber (**Adv Clin Exp Med 2009, 18, 4, 361–368**).

Key words: pseudothrombocytopenia, EDTA-dependent antiplatelet antibodies, pseudoleukocytosis.

Streszczenie

Wprowadzenie. Małopłytkowość rzekoma – m.rz. (pseudotrombocytopenia) jest to zjawisko polegające na zafałszowanym zmniejszeniu liczby krwinek płytkowych spowodowane obecnością przeciwciał, które aglutynacją płytki *in vitro* we krwi pobranej na wersenian dwusodowy (EDTA).
Cel pracy. Ocena kliniczna i serologiczna oraz badania w czasie przeprowadzone na 217 przypadkach m.rz. zdiagnozowanych w Polsce w okresie od 1989–2007 roku.
Materiał i metody. Przeciwciała przeciwpłytkowe EDTA-zależne badano w teście immunofluorescencyjnym (*Platelet Immunofluorescence Test* – PIFT), z użyciem surowicy antyglobulinowej anty-ludzkim IgG i IgM, sprzężonej z izotiocyjanianem fluoresceiny (FITC). Badania przeciwciał w teście PIFT oceniano w cytometrze przepływowym i mikroskopie.
Wyniki. Spośród 217 analizowanych przypadków, m.rz. stwierdzono u 39% osób zdrowych i u 61% chorych najczęściej cierpiących na choroby naczyniowe, infekcyjne, z autoimmunizacji, wątroby i choroby metaboliczne.

Przeciwciała EDTA-zależne wykryto u 97% osób; klasy IgG – 19%, IgM – 34% i IgG+IgM – 44%. Przeciwciała IgG częściej występowały u osób zdrowych niż chorych ($p < 0,01$), a IgM częściej u osób chorych (< 0,025). U 88 osób z m.rz. wykonano powtórne badania (po 1–18 latach, średnio po 4 latach). Wśród tych osób u 31% stwierdzono nową chorobę, u 5 osób pierwotnie zdrowych i u 22 już uprzednio ze stwierdzonymi innymi chorobami. Częstość występowania nowych chorób (z autoimmunizacji, metabolicznych, nowotworowych, naczyniowych i infekcyjnych) nie odbiegała od częstości ich występowania w polskiej populacji ($p > 0,05$). Przeciwciała EDTA zależne utrzymywały się u 81% badanych. Pseudoleukocytozę stwierdzono u 34% osób zdrowych z m.rz.
Wnioski. Obserwacje własne wskazują, że m.rz. nie predysponuje do częstszego występowania wymienionych powyżej chorób. Jest więc prawdopodobne, że przeciwciała EDTA-zależne nie mają znaczenia klinicznego. Niezwykle istotne jest rozróżnienie małopłytkowości rzekomej od prawdziwej, ponieważ pozwala uniknąć niepotrzebnego leczenia. U osób z m.rz.-zależną od EDTA płytki krwi należy liczyć z krwi pobranej na inny antykoagulant niż EDTA lub bezpośrednio po pobraniu krwi w komorze Bürkera (**Adv Clin Exp Med 2009, 18, 4, 361–368**).

Słowa kluczowe: pseudotrombocytopenia, przeciwciała przeciwpłytkowe EDTA-zależne, pseudoleukocytoza.

The characteristic for pseudothrombocytopenia (PTCP) is a false reduction of *in vitro* platelet count in ethylenediaminetetraacetic acid (EDTA) anticoagulated blood. It is due to the presence of antiplatelet autoantibodies clumping platelets in EDTA-collected blood. The size of the clumps sometimes reaches 35 fl (3.5×10^{-14} l) and may be erroneously identified by hematological analyzers as small lymphocytes, thus pseudoleukocytosis [1, 2]. EDTA-dependent platelet autoantibodies were shown to be directed against the 78-kDa GPIIb subunit of the platelet membrane [3]. The most likely hypothesis is that such autoantibodies result from the natural elimination of aged or damaged platelets [4]. It should be mentioned that other spuriously low platelet counts may be related to EDTA due to platelets resettling around white blood cells (satellitism) or their aggregates, but the mechanism responsible for this are not well known [5].

The first description of EDTA-dependent PTCP dates back to 1968 [6]. Its frequency is estimated by various authors at 0.07–0.2% [1, 5–13]. PTCP may appear in both patients and healthy subjects, although it is not fully determined in which diseases it is most often observed and whether the presence of EDTA-dependent antibodies predisposes to any specific disease. To answer these questions, a clinical and immunological analysis was performed as well as a follow-up of PTCP cases diagnosed in Poland within the period of 1989–2007.

Material and Methods

Patients

The study involved 217 PTCP subjects, 152 female and 65 male (median age: 48 years, range: 8–81 years). PTCP was diagnosed in persons with: 1) no observed symptoms of hemorrhagic diathesis, 2) platelet count below 100×10^9/l in an EDTA-anticoagulated blood sample and above 100×10^9/l in blood collected in sodium citrate and/or a platelet count 2–3 times higher in a sodium citrate than in an EDTA sample, 3) platelet clumping in blood smears, and 4) EDTA-dependent antibodies. In the PTCP cases the mean ± *SD* platelet count was 48 ± 33.1 x 10^9/l (range: 0–89 × 10^9/l) in EDTA and 167 ± 65.4 × 10^9/l (range: 73–344 × 10^9/l) in sodium citrate. In 41% (88/217) of the study group the investigations were performed at least twice at 1–18 year intervals (average follow-up time: 4 years). The PTCP cases were divided into two groups: 85 healthy subjects and 132 patients with diseases.

Methods of Platelet Count

Platelet count was performed in blood samples collected into 5% EDTA or 3.2% sodium citrate by a Sysmex automatic analyzer (Model XT-2000I; Kobe, Japan) or a CellDyn (Model 3200; Abbott). In most patients, the time from blood collection to platelet count in the analyzer was approximately 1–4 hours. If the platelets clumped, which also occurred in citrate-anticoagulated blood, the patients were recalled to draw another blood sample and the platelet count was performed immediately following blood collection at 37°C or in blood collected into heparin (5 U/ml) or the platelets were counted directly in a Bürker chamber.

Methods of Anti-Platelet Antibody Detection

EDTA-dependent antibodies were tested by the PIFT (Platelet Immunofluorescence Test) according to von dem Borne et al. [14] using fluorescein isothiocyanate (FITC)-labeled antiglobulin conjugates against human IgG and IgM (Becton Dickinson). The test was performed in both the EDTA and sodium citrate environments at 4°C and 37°C. The results were evaluated by a flow cytometer (Becton Dickinson) and the fluorescence of 10,000 platelets was analyzed using the

Cellquest™ Software computer program. The results were considered positive when the percentage of fluorescent cells exceeded 10% or when the mean fluorescence channel was twice that of the negative control. PIFT results positive in EDTA and negative in sodium citrate were considered as confirmation of the presence of EDTA-dependent antibodies in the serum. These test results were also evaluated microscopically. Platelet clumps in the EDTA sample and no clumps in the sodium citrate sample confirmed the diagnosis of EDTA-dependent pseudothrombocytopenia.

To rule out thrombocytopenia caused by platelet autoantibodies, the serum of patients suspected of PTCP was tested with MAIPA (Monoclonal antibody-specific Immobilization of Platelet Antigens) according to Kiefel et al. [15] using anti-CD41 (GPIIb/IIIa), anti-CD42b (GPIb), and anti-CD49b (GPIa/IIa) (Immunotech) monoclonal antibodies and antiglobulin serum against human IgG conjugated with peroxidase (Jackson ImmunoResearch).

Statistical Analysis

Statistical analysis was performed using [16] Pearson's χ^2 test to compare the percentages of immunoglobulin classes of the platelet EDTA-dependent antibodies and the proportion test to compare the frequencies of the disease groups in the Polish population (2004 data from the Central Bureau for Statistics, CBS, [17]) with those of newly appearing diseases in the PTCP patients. For both statistical methods, a level of $p < 0.05$ was accepted as statistically significant.

Results

Clinical Observations During Primary PTCP Diagnosis

In the total of 217 PTCP cases, 39% (85/217) were healthy subjects and 61% (132/217) were patients with various diseases, the most frequent being vascular (19%), infectious (18%), and autoimmune diseases (17%). The percentages for other diseases (mostly liver related) were below 11% (Table 1).

In 35 of the 152 available PTCP cases (23%), the leukocyte count was elevated (mean: 11×10^9/l; range: $10.3–14 \times 10^9$/l). However, only healthy persons were considered because the elevated leukocyte count was not related to disease. Pseudoleukocytosis was determined in 34% (12/35) of the healthy subjects whose blood smears were available.

Clinical Observations at Follow-Up

Repeat tests were performed in 41% (88/217) of the cases. A new disease appeared in 31% (27/88) of the subjects, in 5 initially healthy and 22 previously ill subjects (Table 2). The difference in disease frequency for both groups was not statistically significant ($p > 0.05$). In Table 3 the frequencies of the new diseases in the PTCP patients are compared with those in the Polish population as reported by the CBS [17]. The differences were not statistically significant ($p > 0.05$).

Retrospectively, treatment was unnecessary in 10% (19/217) of the PTCP cases (cortisone, azathioprine, and platelet concentrate transfusion in two cases). In one case, a surgical procedure was needlessly delayed.

EDTA-Dependent Anti-Platelet Antibodies During Primary Diagnosis of PTCP

EDTA-dependent antibodies were detected in 97% (210/217) of the study group (Table 4). In the remaining 3% (7 cases), no EDTA-dependent antibodies were found, but the PTCP diagnosis was based on the difference in platelet count of blood collected into EDTA and that collected into sodium citrate as well as on the fact that no bleeding symptoms were observed.

The most frequently detected antibodies were IgG + IgM immunoglobulins (44%) in both the healthy subjects and the patients. IgM antibodies appeared alone more frequently in the patients (40%) than in the healthy subjects (24%, $p < 0.025$). On the other hand, IgG antibodies alone were more frequent in the healthy subjects (28%) than in the patients (13%, $p < 0.01$). In 5 cases, both EDTA-dependent antibodies and platelet IgG autoantibodies were found, with anti-GPIb in 2 patients (ITP, Graves-Basedow disease) and anti-GPIIb/IIIa in 3 patients (ITP, Hashimoto diseases, viral hepatitis C).

EDTA-Dependent Antibodies in PTCP Subjects at Follow-Up

Follow-up EDTA-dependent antibody analysis was performed for 88 persons, 1–18 years after the PTCP diagnosis. EDTA-dependent antibodies persisted in 81% of the cases, while no EDTA-dependent antibodies were detected in the remaining 19%. It is worth noting that during follow-up, IgG and IgG + IgM antibodies were observed in 4 subjects who had no EDTA-dependent antibod-

Table 1. Diseases diagnosed in 132 patients at the primary PTCP diagnosis

Tabela 1. Choroby zdiagnozowane u 132 pacjentów, przy rozpoznaniu m.rz.

Group of diseases (Grupa chorób)	Diagnosis – number of patients (Rozpoznanie – liczba pacjentów)
Vascular diseases (Naczyniowe) n = 25 (19%)	essential hypertension (10), atherosclerosis (9), varicoses of lower extremities (2), heart defect (4)
Infectious diseases (Zakaźne) n = 24 (18%)	*Helicobacter pylori* infecton (5), Lyme borreliosis (3), tuberculosis (1), neuroinfection (1), measles (1), adnexitis (1), sepsis (1), toxoplasmosis (4), toxocarosis (1), sinusitis (1), influenza (3), staphylococcal pneumonia(2)
Autoimmune diseases (Autoimmunologiczne) n = 22 (17%)	idiopathic thrombocytopenic purpura (6), systemic lupus erythematosus (5), rheumatoid arthritis (3), Hashimoto's disease (2), Still's disease (1), Cushing's disease (1), juvenile rheumatoid arthritis (1), systemic sclerosis (1), Graves-Basedov disease (1), autoimmune disease under diagnosed (1)
Liver diseases (Wątroby) n = 15 (11%)	viral hepatitis C (10), viral hepatitis B (3), hepatic cirrhosis (2)
Metabolic syndromes (Zespoły metaboliczne) n = 11 (8%)	diabetes (7), hyperlipidemia (3), gout (1)
Neoplastic diseases (Nowotworowe) n = 7 (5%)	breast cancer (2), thyroid cancer (1), pancreas cancer (1), prostate cancer (1), melanoma (1), tumor of the adrenal gland (1)
Arthrosis (Zwyrodnieniowe) n = 6 (5%)	arthrosis (6)
Hematological neoplasms (Nowotwory hematologiczne) n = 5 (4%)	non-Hodgkin's leukemia (4), acute myeloid leukemia (1)
Kidney diseases (Nerek) n = 4 (3%)	chronic renal failure (3) – unknown origin, nephrolithiasis (1)
Other (Inne) n = 12 (9%)	schizophrenia (2), epilepsy (2), pulmonary embolism (1), uterine myomas (1), pancreatitis chronica (1), hypothyroidism (1), hyperthyroidism (1), nodular goiter (1), osteoporosis (1), polyposis of the colon (1)

ies detected at the primary PTCP diagnosis (2 and 2 cases, respectively).

In all PTCP cases, the EDTA-dependent antibodies agglutinated platelets at 4°C, while in 23% they agglutinated platelets at 37°C as well. In 14% of the PTCP cases, antibodies agglutinated platelets not only in EDTA blood, but also in sodium citrate samples (weakly). These were IgM and/or IgG antibodies and in 50% of the cases they clumped platelets not only at 4°C, but also at 37°C, regardless of the immunoglobulin class.

Discussion

So far, the most numerous group, of 112 PTCP subjects, was described by Bizzaro [6] in 1995. The present study included 217 PTCP cases, by far the largest study material reported in the literature. The number of patients exceeded that of healthy subjects. Data for the PTCP groups described in the literature and in the present study are compared in Table 5. Autoimmune diseases were observed to be the most frequent (18–25%). Liver diseases came next (about 10%), with predominant alcoholic hepatic cirrhosis in the literature data [7, 18] and hepatitis C in our study. The frequencies of the other diseases (vascular, neoplastic) in the PTCP patients differ [1, 7, 18–20]. The long-term observations of the present study suggest that PTCP does not predispose to the appearance of new diseases; the frequencies of autoimmune, metabolic, neoplastic, and vascular diseases in the PTCP patients were comparable with those of the Polish population. No such observations were made in the literature; only Berkman et al. [18] reported four cases of PTCP patients with no new disease observed after four years.

Reports regarding immunoglobulin classes of EDTA-dependent antibodies are contradictory

Table 2. New diseases diagnosed during the follow-up of the PTCP subjects

Tabela 2. Nowe choroby zdiagnozowane u osób z m.rz., po upływie czasu od rozpoznania

New diseases (Nowe choroby)	Subjects in follow-up (Osoby po upływie czasu od rozpoznania)		Diagnosis – number of patients (Rozpoznanie – liczba pacjentów)
	healthy n = 24	with diseases n = 64	
Autoimmune (Autoimmunologiczne) n = 8	2 (8.3%)	6 (9%)	rheumatoid arthritis (5), Hashimoto's disease (2), Cushing's disease (1)
Metabolic (Metaboliczne) n = 8	2 (8.3%)	6 (9%)	diabetes (4), hyperlipidemia (3), gout (1)
Neoplastic (Nowotworowe) n = 5	1 (4.2%)	4 (6%)	thyroid cancer (3), cervix cancer (1), prostate cancer (1)
Vascular (Naczyniowe) n = 2		2 (2.5%)	essential hypertension (2)
Infectious (Zakaźne) n = 1		2 (2%)	*Helicobacter pylori* infection
Osteoporosis (Osteoporoza) n = 2		2 (2.5%)	
Cataract (Zaćma) n = 1		1 (2%)	

Table 3. Comparison of the frequencies of new diseases during follow-up of the PTCP subjects and statistical data for the Polish population

Tabela 3. Porównanie częstości pojawienia się nowych zachorowań u osób z m.rz., po upływie czasu od rozpoznania, z danymi statystycznymi występowania tych chorób w populacji polskiej

Group of disorders (Grupa chorób)	PTCP subjects with new diseases (Osoby z m.rz. i nowymi chorobami) n = 88	Disease frequency in the Polish population (Częstotliwość występowania chorób w populacji polskiej) n = 35,248	*P*
Autoimmune diseases (Autoimmunologiczne)	9%	10%	> 0.05
Metabolic syndromes (Zespoły metaboliczne)	9%	6%	> 0.05
Neoplastic diseases (Nowotworowe)	5%	1%	> 0.05
Vascular diseases (Naczyniowe)	2%	3%	> 0.05
Osteoporosis (Osteoporoza)	2%	5%	> 0.05
Cataract (Zaćma)	1%	0.4%	number of patients too low

(Table 6) [7, 19, 21, 22]. IgM antibodies were more frequent than IgG both in our study and in those of Bizzaro [7] and von dem Borne [22]. On the other hand, Pegels et al. [19] and Kabutomori et al. [23] observed IgG antibodies more frequently than IgM antibodies; the data, however, cannot be considered reliable as the studied groups were rather small. The frequencies of combined IgG +

Table 4. Immunoglobulin classes of EDTA-dependent antibodies in PTCP subjects

Tabela 4. Klasy przeciwciał EDTA-zależnych u osób z m.rz.

Antibodies (Przeciwciała)	Number of subjects – % (Liczba pacjentów – %)		
	healthy n = 85	with diseases n = 132	total n = 217
IgG + IgM	37 (44)	59 (45)	96 (44)
IgM	20 (24)	53 (40)	73 (34)
IgG	24 (28)	17 (13)	41 (19)
No antibodies (Brak przeciwciał)	4 (5)	3 (2)	7 (3)

Table 5. Characteristics of PTCP groups (literature data compared with the present study)

Tabela 5. Charakterystyka grup osób z m.rz. (dane z literatury porównano z analizowaną w pracy grupą osób)

No. of cases (Liczba przypadków)	F:M (K:M)	Mean age, years, F/M (Średni wiek, lata K/M)	Mean age, years (Średni wiek – lata)	Healthy/ /with diseases (Zdrowi/ /chorzy)	Diseases – no. of patients (Choroby – liczba pacjentów)	PTCP follow-up years – range (M.rz. po upływie czasu od rozpoznania – lata)	References (Piśmiennictwo)
217	2.3:1	47/49 range: 8–81	48	85/132	vascular 25 (19%) infectious 24 (18%) autoimmune 22(17%) liver 15 (11%) neoplastic 12 (9%) other 34 (26%)	4 (1–18)	present study data
112	1.6:1	range: 6–88	56	54/49	autoimmune 9 (18%) liver 5 (10%) other 35 (75%)	4.7 (6 mos. – 10 years)	Bizarro et al. [7]
52 (data from 4 investigations)	1.4:1	49/63	55	0/52	autoimmune 13 (25%) neoplastic 9 (17%) liver 6 (11.5%) vascular 6 (11.5%) other 18 (35%)	4 years	Berkman et al.[18] Savage et al. [1] Pegels et al. [19] Poskitt& Poskitt [20]
17	2.4:1	47/60	51	11/6	vascular (3) neoplastic (1) autoimmune (1) ITP (1)	nd	Silvestri [30]

IgM antibodies were similar in the present study groups and in those described by other authors [22]. In the present study, IgG antibodies were found more frequently in the healthy subjects than in the patients, in whom IgM antibodies prevailed. No literature reports on this subject are available.

The present study showed that EDTA-dependent antibodies were still present in 81% of the PTCP subjects at follow-up, sometimes for a period of 14–18 years. Similar observations of EDTA-dependent antibody persistence for longer periods were reported by Bizzaro in 2 subjects [7].

In all the PTCP subjects, platelet agglutination *in vitro* due to EDTA-dependent antibodies was observed at 4°C regardless of the immunogloblin class, but in about 23% of the subjects the antibodies also reacted at 37°C. Such reactivity was also observed by Bizzaro [7]. Moreover, in 14% of the subjects of the present study, antibodies were responsible for platelet clumping not only in EDTA, but also in citrate-anticoagulated samples. Following these observations, the most convenient method for an accurate platelet count in PTCP patients seems to be collecting blood into heparin or counting platelets directly in a Bürker chamber. It is worth remembering that pseudoleukocytosis may sometimes appear in PTCP patients [1, 2]. In the present study, pseudoleukocytosis was determined in 34% of the healthy persons whose blood smears were available.

Table 6. Immunoglobulin classes of EDTA-dependent antibodies in PTCP subjects (literature data compared with the present study)

Tabela 6. Klasy przeciwciał EDTA-zależnych u osób z m.rz. (dane z literatury porównano z grupą osób analizowaną w pracy)

Total no. of cases (Suma)	Immunoglobulin classes of EDTA-dependent antibodies no. of cases (%) (Klasy przeciwciał EDTA-zależnych – liczba przypadków)			References (Piśmiennictwo)
	IgM	IgG	IgG + IgM	
210	73 (34%)	41 (20%)	96 (46%)	present study
93	48 (51%)	30 (32%)	12 (13%)	Bizzaro [7]
40	14 (35%)	8 (20%)	96 (44%)	von dem Borne et al. [22]
20	1 (5%)	11 (55%)	8 (40%)	Pegels et al. [19]
10	1 (10%)	3 (30%)	no data	Kabutomori quoted in Breaster [21]

The results of this study confirmed the importance of distinguishing between thrombocytopenia and pseudothrombocytopenia, although they sometimes coexist. There were 5 such PTCP patients in the present study with EDTA-dependent antibodies, but also with antibodies reacting with platelet GPIb and GPIIb/IIIa. To confirm thrombocytopenia, it is also suggested to examine specific platelet autoantibodies in the sera of PTCP persons using the MAIPA test.

In conclusion, PTCP does not seem to predispose to the occurrence of the diseases mentioned in this paper. It is therefore likely that EDTA-dependent antibodies are of no clinical significance. It is worth stressing that an accurate PTCP diagnosis is a safeguard against unnecessary treatment with steroids, platelet transfusions, or splenectomy [11, 18, 23–30]. Unfortunately, about 10% of the PTCP patients in this study had been subjected to such unnecessary treatment. It is therefore always a priority to rule out PTCP in thrombocytopenia cases, especially in patients with no hemorrhagic diathesis**.** From the clinical point of view it is important to rule out pseudothrombocytopenia as the first step in the diagnostic procedure of thrombocytopenias to avoid unnecessary and costly laboratory tests and subsequent treatment. For a proper diagnosis of PTCP, a very simple and quick diagnostic procedure is suggested: 1) evaluation of the platelet count in an EDTA blood sample with an automatic analyzer, 2) microscopic examination for aggregates, and 3) a platelet count in fresh blood in a Bürker chamber.

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