EDTA-Dependent Pseudothrombocytopenia: A Clinical and Epidemiological Study of 112 Cases, With 10-Year Follow-Up

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INTRODUCTION

Patterns of pseudothrombocytopenia (PTCP) detected by automatic instruments for the analysis and counting of blood cells have gained particular attention in recent years, as the use of these machines has become rather widespread in clinical pathology laboratories. PTCP is characterized by a false reduction in the number of platelets in ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood, caused by platelet clumping at room temperature (RT) (Fig. 1A). The phenomenon is due to the presence of antiplatelet autoantibodies that recognize platelet antigens modified by or exposed (cryptoantigens) to the combined action of EDTA and low temperature on the platelet membrane glycoproteins [1-4].

To date there is no clinical evidence that correlates PTCP defective with specific disease (e.g., autoimmune diseases), situations of possible sensitization (i.e., pregnancy or transfusions), or the use of specific drugs; in addition, PTCP has not been associated with hemorrhagic diathesis or platelet dysfunction. Nonetheless, an awareness of this particular condition is necessary, because its lack of recognition may lead subjects with a normal platelet count to be considered, and at times treated, as if they were severely thrombocytopenic.

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In EDTA-dependent PTCp, most of the agglutinins show maximum activity at 4-20°C (cold agglutinins). These autoantibodies may be any of the three major iso-
types (IgG, IgM, or IgA) indifferently mounting κ or λ light chains [2,3,9,14]. An accurate platelet count may be obtained in this case by collecting and examining the blood sample at 37°C. However, EDTA-dependent PTCp, with agglutinins that are reactive both at room temper-
tature and 37°C (wide heat range agglutinins) have been described [15,16], as well as cases of platelet-clumping, 
EDTA-independent but citratedependent, cold antibod-
ies [17,19]. When the clumps are small, a false increase in white cells 
(pseudoaggregation) may be recorded. Indeed, platelet clumps whose size is equal to or slightly larger than a leuco-
cyte, are counted as white cells [7,20,21] (Fig. 1B).

Muntoni et al. [22] in 1975, and then Muntoni et al. [23] in 1981 first studied the frequency of the pheno-
menon, and reported figures of 0.3% and 1.2%, which re-
ferred, however, to a small case series. Since then many 
reports have followed, and this type of alteration is now 
familiar to the clinician pathologist; consequently, it has 
been evaluated more accurately, and at present its fre-
quency is considered to be within the range of 0.09-
0.11% [8,17,21].

The clinical aspects and follow-up of these patients 
have instead been described only sporadically. We re-
viewed the clinical and immunological data of 112 pa-
tients with EDTA-dependent PTCp who were seen in our 
hospital over a 10-year period, from 1985 to 1994. This 
study analyses the clinical and epidemiological aspects, 
as well as the evolution of this phenomenon.

PATIENTS AND METHODS

We studied 112 subjects (43 males and 69 females, 
mean age 35.7 years, range 6-88 years) with EDTA-
dependent PTCp. These cases were detected during rou-
tine hematological studies by the finding of a low platelet 
count (mean value, 72 × 10^9/L) in EDTA-anticoagulated 
blood samples at RT, using the continuous flow auto-
analyzers, Technicon H6000 and H8*2 (Technicon Co., 
Tarrytown, NY), and the appearance of a characteristic 
picture on the histogram indicating the presence of plate-
et clumping. These findings were then confirmed on 
May-Grünwald-Giemsa (MGG)-stained blood films, on 
light microscopy. All patients with suspected PTCp were recalled in order to draw a second EDTA-anticoagulated sample under controlled technical conditions, designed to exclude that platelet clumping might have been caused by microclots due to improper methods of blood drawing and handling. If clumping was still observed on the second examination, the following protocol was carried out: an EDTA-anticoag-
ulated sample and a serum sample were drawn at 37°C.
while a citrate-anticoagulated sample was drawn at RT. In patients who had platelet clumping in EDTA also at 37°C, platelets from capillary blood that had been anticoagulated with ammonium oxalate (Unopette Reagent System, Becton-Dickinson, Rutherford, NJ) were counted in a Barker chamber. All patients were tested more than three times after the diagnosis, and the results were invariably confirmed at subsequent follow-up evaluation.

Among our PTCP cases 70% were outpatients, and the others were hospitalized. In every case, we recorded the patient’s age when the platelet clumping phenomenon had manifested or been observed for the first time. In search of a possible common cause that might explain the appearance of the phenomenon, we recorded the following: type of disease in course at the moment of testing; previous medica history; blood pressure; use of drugs; exposure to toxic substances in the work place; parity in women; blood type and transfusions, if any, and biochemical (i.e., cholesterol, triglycerides, glucose, urea), as well as hematological parameters. Every patient was tested for the presence of rheumatoid factor, cryoglobulins, and red cell cold agglutinins; quantitative immunoglobulins were also assayed. When available, previous hematological reports were examined, with reference to the moment the phenomenon appeared. In the first 34 cases, we also addressed the possible presence of PTCP in other members of the family (first-degree relatives).

**Antiplatelet Antibodies**

The indirect platelet immunofluorescence test (PIFT) was performed on paraformaldehyde-fixed cells according to von dem Borne et al. [24]. Briefly, normal platelets from two healthy donors with blood group O, typed for known platelet-specific antigens, were prepared by differential centrifugation, washed three times in EDTA-phosphate-buffered saline (PBS), pH 7.4, containing 0.1% bovine serum albumin (BSA), and then fixed in 1% (w/v) paraformaldehyde (PFA). The platelets were washed again twice, and resuspended in EDTA-PBS-BSA to a final concentration of 30 × 10⁹/L. Platelets were then incubated with patients’ sera and tested for antibody binding by indirect immunofluorescence using fluorescein isothiocyanate (FITC)-labeled polyclonal rabbit anti-globulin conjugates against human IgG, IgM, IgG, IgA (Dakopatts, Glostrup, Denmark). After another incubation for 30 min at room temperature, the platelets were washed twice, resuspended in a mixture of glyceral-PBS, and examined at the fluorescence microscope.

**Expression of CD5+ B Cells**

Among the healthy subjects with PTCP, 20 were selected for flow cytometry studies of peripheral blood lymphocytes on lithium heparin anticoagulated blood, using a Cytoron cytometer (ORTHO Diagnostic, Raritan, NJ), and the standard procedure [25]. A log forward scatter versus log side scatter cytogram was used to gate the lymphocyte population. Lymphocyte gates were validated using FITC-CD45 (pan-leukocyte) and phycoerythrin (PE)-CD14 (monocyte) monoclonal antibodies. In addition to the usual B (CD19, CD20), T (CD2, CD3, CD4, CD8), and natural killer (NK) (CD56) lymphoid markers, CD5+CD20+ cells were also analyzed by double fluorescence. A minimum of 5,000 cells per sample was analyzed.

**RESULTS**

**Effect of Anticoagulants and Temperature**

The average number of platelets in the 112 patients studied was 72 × 10⁹/L (range 2,000–282,000) in the presence of platelet clumps in samples collected in EDTA at room temperature; about 30% of the patients had platelet counts below 30 × 10⁹/L. In blood samples collected at 37°C, and in samples anticoagulated with ammonium oxalate, the increase in the number of platelets ranged from three- to 20-fold. In three cases, the platelet count at room temperature, even in the presence of platelet clumps, exceeded 200 × 10⁹/L and was thus normal; at 37°C, this figure exceeded 400 × 10⁹/L.

In 22 cases (20%), the phenomenon was EDTA dependent, but clearly temperature independent, having occurred at both room temperature and 37°C; indeed, in three cases it was more pronounced at 37°C. Platelet clumping in citrate-anticoagulated specimens at room temperature was observed in 19 patients, 18 of whom contemporaneously presented EDTA-dependent clumping both at room temperature and at 37°C. Platelet clumping in blood samples anticoagulated with oxalate was not observed.

**Antiplatelet Antibodies**

EDTA-dependent antiplatelet antibodies were demonstrated in 93 of the 112 (83%) PTCP subjects; 30 positive
sera contained platelet reactive antibodies of the IgG class, 48 IgM, and 3 IgA. Two isotypes were present contemporaneously in 22 cases: IgG and IgM in nine cases; IgG and IgA in two, and IgA and IgM in one (Table 1). Citrate-dependent antiplatelet antibodies were detected in 15 cases; 14 IgM, 1 IgA, and no IgG. In four cases of citrate-dependent clumping, no autoantibodies were detected in the patients sera. None of these antibodies showed specificity for leucocyte-platelet alloantigens of the HPA system, since they were reactive with all the platelets on the test panel.

Expression of CD54 B Cells

The lymphocyte immunophenotype in the 20 healthy subjects with PTPC was normal both percentage-wise and in absolute numbers for all the B, T, and NK markers. The percentage of CD54+ B lymphocytes ranged from 0.1% to 0.8% (normal values, 0.1-5.0%) [26].

Clinical Aspects

Among our study subjects, 54 were undergoing routine health examinations, and 49 had various non-autoimmune diseases. Only nine had an autoimmune or a lymphoproliferative disorder: two autoimmune thrombocytopenic purpura (ATTP); two hyperthyroidism; one Hashimoto’s thyroiditis; one Waldenström’s macroglobulinemia; one IgM/A monoclonal gammopathy of undetermined significance (MGUS); one IgG monomya, and one systemic lupus erythematosus (SLE). Hypergammaglobulinemia, present in 7 cases, was monoclonal in the subjects with Waldenström’s macroglobulinemia and MGUS, and polyclonal in five cases with alcoholic liver cirrhosis or chronic hepatitis. Serologic tests for rheumatoid factor, cryoglobulins, and red cell cold agglutinins were negative. Biochemical tests, blood coagulation tests, and hematological parameters (apart from platelet counts) were normal in all healthy individuals or correlated with the clinical picture in subjects with disease. In the set of cases in which we also studied first-degree relatives, no platelet clumping was observed.

Studies regarding drug intake disclosed that 50.5% of patients were receiving therapy with at least one drug when first examined; the most commonly used drugs were blood pressure lowerers (diuretics, β-blockers), benzodiazepines, and oral blood glucose lowerers. Eight patients were taking oral contraceptives, and one was on long-term therapy with oral sodium warfarin for atrial fibrillation.

Age and Gender

By considering the age of the patients when PTPC was first detected, and then subdividing these ages into 10 year groups, we found that PTPC was uniformly present in every age group. Although the female/male ratio was 1.6:1, we observed a clear prevalence of females up to age 50 years, and a slight male prevalence over 50 years of age.

Follow-Up

With the exception of one patient in therapy with anti-D, 111 subjects continued to present this phenomenon regularly in several subsequent control examinations. Mean follow-up was 4.7 years, with a range of 6 months to 10 years. Moreover, by examining the patients’ thrombograms and the clinical records, we detected the presence of thrombocytopenia (probably PTPC) up to 25 and 15 years earlier in three and four cases, respectively; all seven subjects enjoy good health.

DISCUSSION

Autoimmune thrombocytopenia is an immunologically mediated phenomenon caused by the presence of EDTA-dependent cold autoantibodies in serum that cause platelet clumping [1]. The antibodies, usually IgG or IgM, and more rarely IgA, show no platelet group specificity, and are reactive with platelets from normal individuals. They are thus probably directed against cryptic antigens, which are normally undetectable surface antigens, expressed by aged or damaged platelets [2]. Since these antibodies do not react with platelets from patients with Glagmann’s disease [1] that lack the GPIb/IIa complex, it was advanced that the platelet membrane glycoprotein complex GPIb/IIa might be involved in the EDTA-dependent antibody reaction. In some cases, it was shown that autologous specificity is directed against the gpIB fraction [27]. This structure is normally hidden in the GPIb/IIa complex, but becomes accessible to the cold antibody after dissociation of the glycoprotein complex due to the chelating effect of EDTA on calcium ions [28–30], and is also involved in the production of a new form of the GPIb/IIa complex [27,28].

Thus, EDTA might be the cause of the phenomenon, which is further supported by a body of convincing experimental evidence: indeed, several monoclonal antibodies directed against epitopes on the dissociated GPIb/IIa complex, whose binding to platelets was enhanced in the presence of EDTA, have been described [31,32].

Like EDTA, citrate also has a chelating effect on calcium ions, albeit to a lesser extent, and thus might act by modifying the platelet surface with consequent exposure of gpIB/IIa-associated cryptoprotein. Nonetheless, this glycoprotein complex might not constitute the only target for these antibodies, because antibodies directed against a 78-kDa glycoprotein on the platelet membrane have also been described in EDTA-dependent PTPC [33]. Moreover, EDTA-dependent antibodies with a different specificity could exist because EDTA-dependent leucocyte clumping has also been observed [34,35].

The first description of this phenomenon, and subse-
quent studies to specify its prevalence or define the under-
lying pathogenic mechanisms, understandably coincide 
with the introduction and widespread use of automatic 
blood cell counters in the laboratories; the platelet clumps 
that form in PTCP produce warning and/or error flags 
that allow a diagnosis of PTCP if correctly interpreted. 
In this way, we identified 112 cases of EDTA-dependent 
PTCP during the past 10 years, out of a total of 430,000
blood counts that were performed. As our laboratory 
serves an area with almost 100,000 inhabitants, we can 
conclude that at least one blood count/resident was per-
formed in this period. Thus, our prevalence rate would 
be 112/100,000, which is equal to 0.11%, similar to fig-
ures found in the United States by Payne and Pierre [8] 
(0.09%) and Savage [21] (0.11%), in Italy by Vicari et al. 
[36] (0.07%), and in Spain by García Suarez et al. [17] 
(0.11%).

Ninety-three of the 112 subjects (83%) had detectable 
serum levels of antplatelet antibodies, most of which were 
IgM or IgG. IgA were found in three cases. In the 
22 cases in which platelet clumping was also observed 
at 37°C, 16 had IgM antibodies, two had IgG, and one 
had IgA; antplatelet antibodies were not detectable in 
the other three cases.

Platelet clumping occurred in sodium citrate-anticoagu-
lated blood in 19 cases (16.9%). Here as well, the antibodies 
were most frequently IgM (14 cases), with no cases of IgG 
and one of IgA; in four cases, antibodies were not found.

It is noteworthy that 18 of these 19 cases were also positive 
in EDTA at 37°C, and in 93% IgM antibodies were present, 
thus confirming previous reports [9] that citrate-dependent 
antibodies are almost always IgM.

Our findings thus show that all the major antibody 
classes are involved in the platelet clumping phenomenon; 
IgM antibodies are much more frequent than IgG, and IgA 
are rarely involved. When this event also occurs at 37°C 
(20% of our cases, IgM antibodies are almost always pres-
ent. Citrate-dependent antibodies are also almost invari-
ably IgM, and, at least in our series, are probably the same 
antibodies responsible for EDTA-dependent clumping at 
37°C. Therefore, based on our experience, the use of ci-
trate-anticoagulated samples is contraindicated. The most 
reliable and rapid method for obtaining accurate platelet 
counts in PTCP patients is to collect and examine EDTA 
blood at 37°C. On the rare cases that show clumping at 37°C as well, blood should be collected in ammonium oxal-
ate, and platelet counted in a Burker chamber.

The meaning of antplatelet antibody presence in both 
healthy subjects and persons with various diseases, how-
ever, remains to be clarified. The most likely hypothesis 
is that we are dealing with natural antautoantibodies with 
no pathological significance, and a physiological role in 
the removal of senescent circulating platelets [2,3,7].

Several studies during the past 25 years have described 
natural antautoantibodies in the sera of healthy subjects [38-
44]. It was shown that natural antautoantibodies, which are 
the product of normal germline genes [45], actively partici-
pate in the general homeostasis of the organism with the 
clearance of altered self-components [46,47]; furthermore, 
in normal individuals, at least 20% of all the immu-
oglobulins correspond to polyclonal natural autoon-
tibodies able to recognize self-structures [47-49]. Indeed, 
these natural antautoantibodies would be directed against 
self-antigens that are more frequent on the platelet membrane surface that are exposed follow-
ing an alteration in the cell membrane). This alteration 
could be of an aging process, or, in the case of PTCP, the product of EDTA action. Therefore, only the quantity of natural antautoantibodies directed against 
autologous platelets would be greater in subjects with 
PTCP, compared to other individuals.

None of the family members of our PTCP patients 
showed clumping. Therefore, unlike the familial occur-
rence of antautoantibodies in first-degree relatives of patients 
with autoimmune disease, there is no familial tendency 
for natural antautoantibody occurrence; this indicates that 
the finding of antautoantibodies in an apparently healthy 
subject does not mean a higher risk of the future develop-
ment of an overt autoimmune condition [50,51].

As CD5+ B cells are responsible for the production of 
low-avidity polyclonal antautoantibodies without 
antigenic challenge [47,49,52,53], we studied 20 of our 
healthy patients to see whether CD5 + B cell expression 
was increased. Very low CD5+ B-cell percentages that 
were less than 1% of the total lymphocytes were found, 
thus demonstrating that natural antautoantibodies are not 
the product of a clonal expansion.

Clinical and follow-up analyses disclosed no correla-
tion between PTCP and state of health, or type of disease. 
Nonetheless, the two cases of AHT associated with PTCP 
are interesting, because this previously described combi-
nation [2,54], further lowers the platelet count, and pres-
ents a characteristic picture of PTCP in true thrombocyto-
penia [55]. This possibility should be kept in mind for a 
correct evaluation of the platelet count in subjects who 
are already thrombocytopenic.

Another interesting observation was antplatelet activi-
ty IgM antibodies in two patients with monoclonal 
gammopathy (MG). Although it was clearly demonstrated 
that sera from patients with MC may have antibody activ-
ity against a large number of self-antigens [56-58], cases 
of PTCP are extremely rare; it is addition to our two pa-
tients, only one other has been described [59].

Fetomaternal incompatibility due to erythrocyte anti-
gens occurred in a group O Rh-negative patient who had 
given birth to a group O Rh-positive male child. PTCP 
first appeared in this patient a few days after the immuno-
 prophylactic administration of 250 µg of human anti-D 
immunoglobulin (Pertolbin, Immuno AG, Vienna, Aus-
tria). The antplatelet antibody involved was IgM class,
and was active both at room temperature and at 37°C, but not in a DMSO. This patient was checked every month, and the picture was unchanged for 5 months after this time, it disappeared, and is still absent after 4 years. As Rh antigens are not present in the platelet membrane [60], we might have been dealing with either a cross-reaction between other erythrocytic and platelet antigens, or more likely, antiplatelet alloantibodies present in the immune serum (and, therefore, natural antibodies in the donor serum).

Also worthy of note was the case of a patient on oral anticoagulant therapy (warfarin); numerous examinations in the months preceding the start of warfarin administration documented the absence of PHC whose onset exactly coincided with the start of the anticoagulant therapy. Because of ethical considerations, we did not interrupt this therapy, and Ibs cannot determine if the PHC, which is still present, is indeed related to it.

In reference to follow-up, it is noteworthy that in some of our patients PT/INR presence was documented for years, and in more cases was considered a chronic APTT. Follow-up in four and three of these subjects exceeded 15 and even 20 years, respectively.

In one of the third group of subjects, it was definitely determined that PHC had not been present in previous years but had appeared suddenly with no apparent cause. Nonetheless, excluding the patient undergoing ant-D prophylaxis, after PHC appeared, in none of our cases did it disappear with time; all subjects continue to present this phenomenon at every checkup, even if the entity of the clumping, and subsequently the platelet count, varies in intensity. Indeed, there is general agreement regarding the persistence of the phenomenon, even though none of the reported follow-up evaluations exceed 2 years.

This study, which comprises a vast epidemiological survey and the longest follow-up on the occurrence of EDTA-dependent PHC, demonstrates that the phenomenon is absolutely not correlated with gender, age, and age of onset, nor a particular disease, hemostasis alterations (thrombocytopenia or thrombosis) or ingestion of specific drugs. Follow-up management, which exceeds 20 years in some cases, confirms that EDTA-dependent PHC is an in vivo phenomenon with no pathological significance and is related to the presence of antiplatelet antibodies that are most likely recognize plasmin membrane antigens that have been altered or exposed due to the effect of EDTA. Further studies and a greater understanding of the mechanisms that regulate natural auto-antibody synthesis and function will explain other aspects of this interesting phenomenon.

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