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Peripheral blood lymphocyte phenotype analysis in immune thrombocytopenic purpura

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OBJECTIVE **Differing dominant T-cell and B-cell pathophysiological mechanisms may be involved in immune thrombocytopenic purpura (ITP). In this study phenotype analysis was made of peripheral blood lymphocyte abnormalities and correlation explored between circulating lymphocyte subtypes and age, sex, serological findings and disease course.** METHOD **A retrospective study was conducted of 20 adults and 3 children with ITP whose lymphocyte phenotype analysis data were compared with those of 20 age- and sex-matched healthy volunteers.** RESULTS **No differences were observed between patients with well-responding and relapsing ITP. Negative correlation was demonstrated between $CD20^+/CD23^+$ B-cell levels and the number of relapses per year and positive correlation between high $CD19^+$ and $CD22^+$ levels and the need for splenectomy. $CD5^+$ and $CD7^+$ T-cell levels were inversely related with the detection of ANA and IgG anti-cardiolipin autoantibodies. Patients aged above 60 years had significantly lower levels of $CD2^+$ and $CD3^+$ T-cells and higher levels of $CD5^+/CD19^+$ co-expression. Finally, $CD19^+$, $CD20^+$, $CD22^+$ B-cell levels, $CD19^+/CD79b^+$, $CD19^+/CD25^+$ and $CD20^+/CD23^+$ markers, and $Fmc7^+/CD11c^+$ co-expression were all significantly raised in patients with ITP.** CONCLUSIONS **B-lymphocyte abnormalities and age-related T-cell defects may be implicated in the pathogenesis and outcome of ITP.**

Ανάλυση του φαινότυπου λεμφοκυττάρων του περιφερικού αίματος στην αυτοάνοση θρομβοπενική πορφύρα

Περίληψη στο τέλος του άρθρου

Key words

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Immune thrombocytopenic purpura (ITP) is a common acquired hematological disorder. Its incidence in adults is estimated at 50 cases per million per year, with double the incidence in females than in males.[1] The incidence appears to increase with age, while the gender difference is eliminated above the age of 60 years.[2] The onset of ITP is often insidious,[1] and there are no specific criteria that establish the diagnosis, which is based mainly on exclusion of alternative causes of thrombocytopenia, and on the patient's history, physical examination, blood count and examination of the peripheral blood smear.[1,3,4]

ITP is characterized by accelerated platelet consumption, mediated by autoantibodies, and mucocutaneous bleeding. The thrombopoietin level and the megacaryocyte mass remain normal in the majority of cases,[1] although megacaryopoiesis may also be affected by autoantibodies.[5]

Two forms of the disorder have been recognized, the acute form, with thrombocytopenia that lasts less than 6 months, and the chronic form. The chronic form is markedly more common in adult patients and is often relapsing and refractory to therapy, with a risk of fatal bleeding of up to 5%.[1,6] It is still impossible to predict which patients will develop chronic ITP, although a pathophysiological mechanism different from that of acute ITP is thought to be responsible.[7] It has been reported that chronic ITP is associated with Thl cell defects,[5,8–10] abnormal platelet

reactive T-cell and B-cell proliferation,[11,12] upregulation of CD86+ macrophages[10] and changes in the immune system with advancing age.[7] Overall, ITP appears to be a heterogeneous disorder in which differing T-cell dominant and B-cell dominant pathophysiological mechanisms may be operating.[13] The aim of this study was to determine, using immunophenotype analysis, specific lymphocyte subsets in the peripheral blood of patients with ITP in comparison with normal subjects, and explore their possible correlation with age, gender, serological findings and outcome of the disease.

MATERIAL AND METHOD

Patients

Twenty adults with ITP, 15 females and 5 males, with a median age of 32.5 years (range 18–69 years), and 3 children, 2 boys and 1 girl, with a median age of 14 years (range 10–16 years) were studied retrospectively. All of the patients had been treated in the same department between 2002 and 2008 and their median follow-up was 38.3 months (range 2–144 months). Twenty healthy adult age- and gender-matched volunteers, 13 females, 7 males, median age 27 years (range 22–68 years), were enrolled as control group.

All the patients with ITP met the standard diagnostic criteria: Platelet count of less than 80×10^9/L, normal or increased number of megacaryocytes on bone marrow aspiration and no other clinically apparent cause of thrombocytopenia. Patients with positive antinuclear and anti-cardiolipin autoantibodies, but with no evident underlying disorder were also enrolled in the study.

Assays

All the serological tests had been conducted at diagnosis to eliminate acute viral infections or other autoimmune disorders as the cause of thrombocytopenia. In addition, all the patients had undergone peripheral blood flow cytometric immunophenotype analysis in the same hospital laboratory to exclude other hematological disorders as the cause of thrombocytopenia, with the exception of one of the children who had first been admitted to a pediatric hospital, from which his medical records were obtained. The phenotype analysis had been conducted either at diagnosis or during relapse after a minimum of 3 months without administration of any drug.

All results expressed as percentages were converted into absolute counts using the absolute lymphocyte count (ALC) on the day of the examination (this does not apply for co-expressions $CD5^+/CD19^+$, $CD20^+/CD23^+$, $Fmc7^+/CD11c^+$, $CD19^+/CD79b^+$, $CD10^+/CD22^+$, $CD19^+/CD25^+$, $CD43^+/CD19^+$). Percentages of circulating lymphocyte subsets and natural killer (NK) cells were measured in 20 healthy volunteers by flow cytometric analysis using a panel of six fluorescence monoclonal antibodies (FACSCanto, BD Biosciences, San Jose, California) and converted to absolute counts using the ALC.

Statistical analysis

All continuous variables were expressed as mean±2SD (standard deviation) of the controls and classified on an ordinal scale. Differences in the frequency between groups were compared using the Chi-squared test, supported by the Monte Carlo procedure, and Fisher's exact test, as well as the Mann-Whitney U-test, when applicable. The data were analyzed using the Statistical Package for Social Sciences (SPSS), version 15.0 and Minitab 12 software. Probability values of $p<0.05$ were considered to be statistically significant.

RESULTS

Case control study

The levels of $CD19^+$, $CD20^+$ and $CD22^+$ B-cells were significantly higher in patients with ITP than in healthy subjects. $CD19^+/CD79b^+$, $CD19^+/CD25^+$ and $CD20^+/CD23^+$ (activated and mature B-cell markers) were also significantly higher in the patients, as was the co-expression of $Fmc7^+/CD11c^+$ ($p=0.01$). No other statistically significant differences were demonstrated. No differences between the two groups were observed in NK cells and suppressor T-cells (tab. 1, figures 1, 2).

Correlations

Concerning the course of the disease, no differences were observed between patients with well-responding and relapsing ITP. The number of relapses per year was lower in patients with a raised $CD2O^+/CD23^+$ rate ($p=0.024$). A significant number of those with high $CD19^+$ and $CD22^+$ levels were eventually splenectomized ($p<0.05$). Comparing the patients' immunophenotype analysis findings with

Table 1. Statistically significant differences in lymphocyte immunophenotype findings between patients with immune thrombocytopenic purpura and control subjects.

Markers	p value
$CD19^+$	0.014
$CD20^+$	0.001
$CD22^+$	0.027
$CD20^+/CD23^+$	0.001
$CD19^+/CD79b^+$	0.000
$FMC7^+/CD11c^+$	0.001
$CD19^+/CD25^+$	0.028

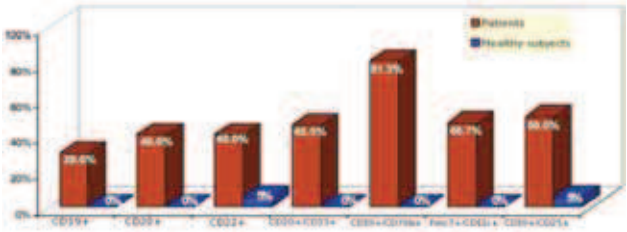

Figure 1. Comparison between patients with immune thrombocytopenic purpura with increased levels of B-cell markers (mean±2SD [standard deviation] of controls) and healthy control subjects.

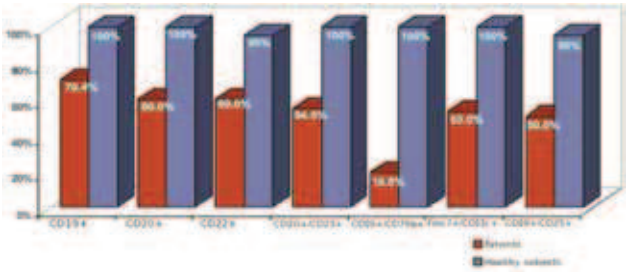

Figure 2. Comparison between patients with immune thrombocytopenic purpura with levels of B-cell markers within the normal range (mean±2SD [standard deviation] of controls) and healthy control subjects.

their serological tests at diagnosis, negative correlation was established between $CD5^+$ and $CD7^+$ cell levels and positive anti-nuclear antibodies (ANA) and IgG anti-cardiolipin ($p<0.05$). Patients aged above 60 years had significantly lower levels of $CD2^+$ and $CD3^+$ and higher rates of $CD5^+$/$CD19^+$ co-expression. The mean age of those with a high rate of $CD5^+$/$CD19^+$ was 66.5 years. No differences were observed between male and female patients (figures 3, 4).

DISCUSSION

These findings suggest that patients with ITP present

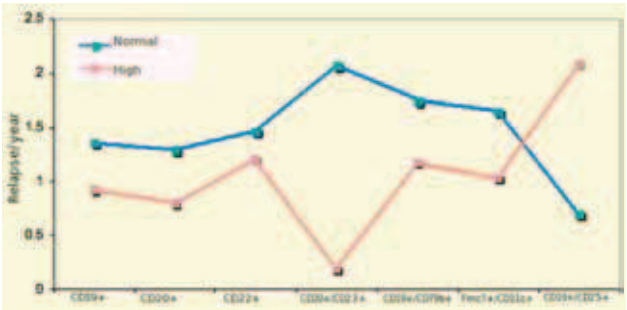

Figure 3. Number of relapses per year in patients with immune thrombocytopenic purpura with high and normal (mean±2SD [standard deviation] of controls) levels of B-cell markers.

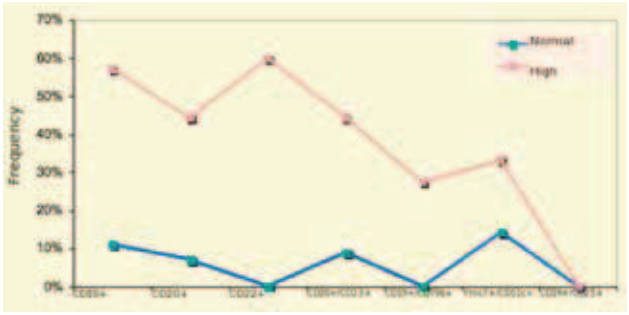

Figure 4. Frequency of splenectomy in patients with immune thrombocytopenic purpura with high and normal (mean±2SD [standard deviation] of controls) levels of B-cell markers.

an increase in the mature and activated B-cell population compared with healthy control subjects. This is consistent with the findings of a study which showed that $CD19^+$/$CD40^+$ and $CD19^+$/$CD40^+$/$CD19^+$ B-cells were significantly higher in patients with ITP.[11] Elsewhere $CD19^+$ B-cells have also been found to be upregulated in ITP.[8] The above studies and recent works from China[5] and Canada[9] support the conclusion that T-cells, especially $CD4^+$ and activated $CD3^+$ cells, play a major role in the pathogenesis of the disease, especially its chronic form. The idea that T-cell activation is the critical event which determines the production of autoantibodies against platelets is widely accepted,[10,12] although B-cell specific therapeutic agents (rituximab) are very effective for many patients with ITP, leading to a rapid and long-lasting response.[13] Although recent studies suggest that NK cells also play a role in the biology of ITP,[14–16] in the present study no significant differences in NK cells were found between patients with ITP and healthy control subjects.

The significantly higher expression of $Fmc7^+$/$Cd11c^+$ (a marker of B-lymphoproliferative disorders) in patients with ITP with no clinically or otherwise apparent underlying hematological disorder remains to be investigated, although it suggests that ITP may occur when an underlying B-cell disorder is present but not yet recognizable.

Patients with refractory ITP who had raised rates of $CD20^+$/$CD23^+$ (the mature B-cell phenotype) had a significantly lower number of relapses per year, while almost 60% of those with raised $CD19^+$ and $CD22^+$ levels were eventually splenectomized because of the resistance of the disease to conventional drug therapy. There were no post-operative differences in the course of the disease.

The inverse correlation found between $CD5^+$ and $CD7^+$ cells and positive antinuclear antibodies or anti-cardiolipin IgG is consistent with the overall changes in the immune

system that have been described in autoimmunity[17] and might also suggest an autoimmune background to the ITP, although patients with such findings and no other clinically evident cause of thrombocytopenia were included at diagnosis.[3] Negative correlation was also observed between T-cell levels and age, and positive correlation between age and over-expression of $CD5^+/CD19^+$, a marker often found in patients with autoimmune disorders. These findings are consistent with recent evidence on the relationship between advancing age and increase in the incidence of autoimmunity.[18,19]

In conclusion, the levels of activated and mature B-cells and $Fmc7^+/CD11c^+$ cells were significantly raised in patients with ITP compared with healthy control subjects. Significant correlation was also demonstrated between B-lymphocyte subtypes and a refractory course of the disease, while T-lymphocyte abnormalities were mainly associated with advancing age. These findings suggest that B-lymphocyte abnormalities and age-related T-cell defects may be implicated in the pathogenesis and outcome of ITP. Additional studies will be useful in the elucidation of the biology of ITP, the prediction of the outcome and the possible identification of biomarkers for appropriate targeted therapy.

ΠΕΡΙΛΗΨΗ

Ανάλυση του φαινότυπου λεμφοκυττάρων του περιφερικού αίματος στην αυτοάνοση θρομβοπενική πορφύρα

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ΣΚΟΠΟΣ Ανάλυση του φαινότυπου των λεμφοκυττάρων του περιφερικού αίματος σε ασθενείς με αυτοάνοση ιδιοπαθή θρομβοπενική πορφύρα (ΙΘΠ) και διερεύνηση των πιθανών συσχετίσεων των υποτύπων των λεμφοκυττάρων ανάλογα με την ηλικία, το φύλο, τα ορολογικά ευρήματα και την πορεία της νόσου. **ΥΛΙΚΟ-ΜΕΘΟΔΟΣ** Είκοσι ενήλικες και 3 παιδιά μελετήθηκαν αναδρομικά. Τα δεδομένα της ανάλυσης του φαινότυπου συγκρίθηκαν με τα δεδομένα υγιών εθελοντών, ηλικίας 20 ετών και ως προς το φύλο. **ΑΠΟΤΕΛΕΣΜΑΤΑ** Δεν βρέθηκαν διαφορές μεταξύ ατόμων με καλή ανταπόκριση και υποτροπή της νόσου. Διαπιστώθηκε αρνητική συσχέτιση μεταξύ των επιπέδων των $CD20^+/CD23^+$ Β-λεμφοκυττάρων και του αριθμού των υποτροπών ανά έτος, καθώς και θετική συσχέτιση μεταξύ των αυξημένων επιπέδων $CD19^+$ και $CD22^+$ λεμφοκυττάρων και της συχνότητας της σπληνεκτομής. Υπήρχε αντίστροφη συσχέτιση των επιπέδων των $CD5^+$ και $CD7^+$ Τ-λεμφοκυττάρων με τη συχνότητα θετικών ANA και IgG αυτοαντισωμάτων κατά της καρδιολιπίνης. Οι ασθενείς ηλικίας >60 ετών είχαν σημαντικά χαμηλότερα επίπεδα $CD2^+$ και $CD3^+$ Τ-λεμφοκυττάρων, καθώς και υψηλότερα επίπεδα λεμφοκυττάρων με συνέκφραση $CD5^+/CD19^+$. Τελικά, τα επίπεδα των $CD19^+$, $CD20^+$, $CD22^+$ Β-λεμφοκυττάρων, καθώς και η συνέκφραση των δεικτών $CD19^+/CD79b^+$, $CD19^+/CD25^+$ και $CD20^+/CD23^+$ αλλά και η συνέκφραση $Fmc7^+/CD11c^+$ βρέθηκαν σημαντικά αυξημένα στους ασθενείς με ΙΘΠ. **ΣΥΜΠΕΡΑΣΜΑΤΑ** Τα ευρήματα υποθέτουν ότι οι διαταραχές των Β-λεμφοκυττάρων, καθώς και τα ελλείμματα των Τ-λεμφοκυττάρων που σχετίζονται με την ηλικία, μπορεί να ευθύνονται για την παθογένεια και την πορεία της νόσου.

Λέξεις ευρετηρίου: Ανοσοφαινότυπος, Θρομβοπενία, Ιδιοπαθής θρομβοπενική πορφύρα, ΙΘΠ

References

1. CINES DB, BLANCHETTE VS. Immune thrombocytopenic purpura. *N Engl J Med* 2002, 346:995–1008
2. FREDERIKSEN H, SCHMIDT K. The incidence of idiopathic thrombocytopenic purpura in adults increases with age. *Blood* 1999, 94:909–913
3. GEORGE JN, WOOLF SH, RASKOB GE, WASSER JS, ALEDORT LM, BALLEM PJ ET AL. Idiopathic thrombocytopenic purpura: A practice guideline developed by explicit methods for the American Society of Hematology. *Blood* 1996, 88:3–40
4. KUWANA M, KURATA Y, FUJIMURA K, FUJISAWA K, WADA H, NA-

GASAWA T ET AL. Preliminary laboratory based diagnostic criteria for immune thrombocytopenic purpura: Evaluation by multi-center prospective study. *J Thromb Haemost* 2006, 4:1936–1943
5. ZHOU B, ZHAO H, YANG RC, HAN ZC. Multi-dysfunctional pathophysiology in ITP. *Crit Rev Oncol Hematol* 2005, 54:107–116
6. SCHOONEN WM, KUCERA G, COALSON J, LI L, RUTSTEIN M, MOWAT F ET AL. Epidemiology of immune thrombocytopenic purpura in the General Practice Research Database. *Br J Haematol* 2009, 145:235–244
7. CULIĆ S, LABAR B, MARUSIĆ A, SALAMUNIĆ I. Correlations among age, cytokines, lymphocyte subtypes, and platelet counts in autoimmune thrombocytopenic purpura. *Pediatr Blood Cancer* 2006, 47(Suppl 5):671–674
8. SEMPLE JW, FREEDMAN J. Increased antiplatelet T helper lymphocyte reactivity in patients with autoimmune thrombocytopenia. *Blood* 1991, 78:2619–2625
9. SEMPLE JW. T cell and cytokine abnormalities in patients with autoimmune thrombocytopenic purpura. *Transfus Apher Sci* 2003, 28:237–242
10. SEMPLE JW, LAZARUS AH, FREEDMAN J. The cellular immunology associated with autoimmune thrombocytopenic purpura: An update. *Transfus Sci* 1998, 19:245–251
11. MEABED MH, TAHA GM, MOHAMED SO, EL-HADIDY KS. Autoimmune thrombocytopenia: Flow cytometric determination of platelet-associated CD154/CD40L and CD40 on peripheral blood T and B lymphocytes. *Hematology* 2007, 12:301–307
12. SEMPLE JW, MILEV Y, COSGRAVE D, MODY M, HORNSTEIN A, BLANCHETTE V ET AL. Differences in serum cytokine levels in acute and chronic autoimmune thrombocytopenic purpura: Relationship to platelet phenotype and antiplatelet T-cell reactivity. *Blood* 1996, 87:4245–4254
13. BENNETT CM, DE JONG JL, NEUFELD EJ. Targeted ITP strategies: Do they elucidate the biology of ITP and related disorders? *Pediatr Blood Cancer* 2006, 47(Suppl 5):706–709
14. CATANI L, FAGIOLI ME, TAZZARI PL, RICCI F, CURTI A, ROVITO M ET AL. Dendritic cells of immune thrombocytopenic purpura (ITP) show increased capacity to present apoptotic platelets to T lymphocytes. *Exp Hematol* 2006, 34:879–887
15. GARCIA-SUAREZ J, PRIETO A, REYES E, MANZANO L, MERINO JL, ALVAREZ-MON M. Severe chronic autoimmune thrombocytopenic purpura is associated with an expansion of CD56+ CD3-natural killer cells subset. *Blood* 1993, 82:1538–1545
16. YOSHIMURA C, NOMURA S, NAGAHAMA M, OZAKI Y, KAGAWA H, FUKUHARA S. Plasma-soluble Fas (APO-1, CD95) and soluble Fas ligand in immune thrombocytopenic purpura. *Eur J Haematol* 2000, 64:219–224
17. LEMOINE S, MORVA A, YOUINOU P, JAMIN C. Regulatory B cells in autoimmune diseases: How do they work? *Ann N Y Acad Sci* 2009, 1173:260–267
18. BOREN E, GERSHWIN ME. Inflamm-aging: Autoimmunity, and the immune-risk phenotype. *Autoimmun Rev* 2004, 3:401–406
19. LARBI A, FÜLÖP T, PAWELEC G. Immune receptor signaling, aging and autoimmunity. *Adv Exp Med Biol* 2008, 640:312–324

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