PERGAMON

Transfusion Science 22 (2000) 19–27

TRANSFUSION SCIENCE

www.elsevier.com/locate/transci

Comparison of platelet immunity in patients with SLE and with ITP

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Received 18 October 1999; accepted 30 November 1999

Abstract

Idiopathic thrombocytopenic purpura (ITP) is characterized by the development of a specific anti-platelet autoantibody immune response mediating the development of thrombocytopenia. Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of a wide variety of autoantibodies. In 15–20% of SLE cases, patients develop thrombocytopenia which appears to be autoimmune in nature (SLE-TP). To better understand the pathogenesis of the thrombocytopenia associated with SLE, we investigated the overlapping platelet and cellular immune features between SLE and ITP. Thirty-one patients with SLE, eight with SLE-TP, and 17 with ITP, were studied and compared to 60 healthy controls. We evaluated platelet-associated IgG, platelet microparticles, reticulated platelets, platelet HLA-DR expression, in vivo cytokine levels, lymphocyte proliferation, and the T lymphocyte anti-platelet immune response in these patients. Patients with SLE-TP and those with ITP had increased platelet-associated IgG, an increased percentage of platelet microparticles, a higher percentage of reticulated platelets and larger platelets, suggesting antibody-mediated platelet destruction and increased platelet production. More than 50% of patients with ITP had increased HLA-DR on their platelet surface whereas subjects with SLE-TP did not. Analysis of serum cytokines demonstrated increased levels of IL-10, IL-15 and TNF-α in patients with SLE, but in those with ITP, only increased levels of IL-15 were seen, no increases in any of these cytokines were observed in patients with in SLE-TP. The ability of lymphocytes to proliferate in response to phorbol myristate acetate (PMA) stimulation was increased in SLE-TP, but was normal in both SLE and ITP. Lymphocytes from subjects with ITP displayed an increased ability to proliferate on exposure to platelets, in contrast, those with SLE-TP did not. While the number of subjects evaluated with SLE-TP was small, these data reveal a number of differences in the immunopathogenesis between SLE-TP and ITP.

Keywords: Systemic lupus erythematosus; Idiopathic thrombocytopenic purpura; Platelets; Cytokines

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0955-3886/00/$ - see front matter
PII: S0955-3886(00)00004-7

1. Introduction

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by the production of autoantibodies to a number of self-antigens, including nuclear, cytoplasmic and cell surface autoantigens. Although the mechanism(s) responsible for initiating the disease is unknown, it is well known that immune abnormalities at the level of B lymphocytes, T lymphocytes, cytokine production, and of antigen presenting cells (APC) have been described in both human and animal models of SLE.

Idiopathic chronic autoimmune thrombocytopenic purpura (ITP) in adults is an autoimmune-mediated bleeding disorder in which platelets are opsonized by platelet autoantibodies and prematurely destroyed in the reticuloendothelial system. Chronic ITP, in the absence of a secondary autoimmune disease, is considered to be a true autoimmune syndrome. Several studies have demonstrated defects in cellular immunity in patients with ITP [1,2]; these include increased expression of MHC class II antigens on platelets [3,4], altered in vivo and in vitro cytokine expression [4–7], and defects in several different lymphocyte functions [5,8–10].

Approximately 15–20% of patients with SLE develop thrombocytopenia (TP) and thrombocytopenia in SLE are associated with an increased risk of mortality [11]. It is not currently known if patients with SLE-TP have SLE and ITP as separate disorders or whether such patients have SLE with antibodies that coincidentally cross-react with platelets and cause TP. Both SLE and ITP are characterized by abnormalities in B cell, T cell and cytokine production.

In an attempt to determine if SLE and ITP have common immunological attributes, we directly compared platelet and immunologic profiles of patients with SLE and ITP in the same study. We observed that while ITP and SLE-TP did have some common platelet specific features, there were significant differences in some underlying immunologic parameters which may indicate that SLE-TP and ITP are different syndromes.

2. Materials and methods

2.1. Patients

With informed consent, venous whole blood from all subjects was taken into heparin, EDTA and non-treated vacutainer tubes for isolation of peripheral blood mononuclear cells (PBMC), platelets, and determination of serum cytokines, respectively. The samples were all tested and blinded as to the category/diagnosis of the subject. All patients were adults. ITP was defined as thrombocytopenia (platelet count $< 150 \times 10^9\ l^{-1}$) with normal or increased marrow megakaryocytes, in the absence of splenomegaly and secondary immune or non-immune abnormalities that could account for the thrombocytopenic state. SLE was defined according to the 1982 revised American Rheumatism Association criteria [12], and SLE-TP was defined as any patient with SLE who had a platelet count $< 150 \times 10^9\ l^{-1}$ on the day of analysis. The samples were all tested and blinded as to the category/diagnosis of the subject.

2.2. Reagents

Ionomycin and alkaline phosphatase-streptavidin were obtained from Calbiochem (La Jolla, CA). Thiazole Orange (TO) was purchased from Becton–Dickinson (San Jose, CA). Fluorescein isothiocyanate (FITC)-labeled anti-CD41 and anti-CD42b were obtained from Immunotech (Coulter Electronics, Westbrooke, ME) and FITC-labeled antihuman IgG from Organon Teknika (Scarborough, ON) or Immunocorp Sciences (Montreal, PQ). Monoclonal FITC- and phycoerythrin (PE)-labeled isotype controls, and PE-anti-HLA-DR reagents were obtained from Becton–Dickinson. The 0.8 μm polystyrene latex marker beads used to discriminate platelets from platelet microparticles were purchased from Sigma (St. Louis, MO). Interleukin IL-2 was purchased from Genzyme (Cambridge, MA) and IL-4, IL-10, IL-15, TNF-α and antihuman IL-4 (clone 8D4-8) were from Pharmingen (Mississauga, ON). Mouse antihuman IL-2 antibody (Cat# 80-3638-02) and biotin-labeled rabbit antihuman IL-2 antibody (Cat# 80-3637-02) were from Genzyme.

Biotin-labeled rat antihuman IL-4 (clone MP4-25D2), rat anti-IL-10 (clone JE53-12G8), biotin-labeled rat anti-IL-10 (clone JE53-12G8), mouse anti-IL-15 (clone G243-935), biotin-labeled mouse anti-IL-15 (clone G243-935), mouse anti-tumour necrosis factor (TNF)-α (clone Mab1) and biotin-labeled mouse anti-TNF-α (clone MAb11) were from Pharmingen. All other reagents were obtained from Sigma.

2.3. Flow cytometric analysis of platelets

HLA-DR quantitation on platelets was performed as previously described [4]. In brief, whole blood was incubated with FITC- or PE-labeled antibodies for 30 min in the dark at 22°C, diluted and analyzed by a FACS can flow cytometer (Becton–Dickinson) equipped with an argon ion laser operating at 15 mW. Platelets were acquired using forward scatter (FSC; reflecting size) and a fluorescence (FL1) threshold set on the lowest fluorescent channel of positive reactivity with FITC-anti-GPIb (CD42b) or-GPIIbIIIa (CD41a). Ten thousand GPIb- or GPIIbIIIa-positive events were acquired and analysis markers were set based on appropriate isotype controls. Reticulated platelets were enumerated by staining with TO as previously described [13]; analysis markers were set on TO-stained normal GPIb-positive platelets to give <1% TO-positive events. Platelet-derived microparticles were distinguished from intact platelets based on their reactivity with the anti-CD41 antibody and were smaller than 0.8 μm polystyrene latex beads.

2.4. Cytokine analysis

Sera from the patients and normal controls were tested for the presence of IL-2, IL-4, IL-10, IL-15 and TNF-α by solid-phase enzyme immunoassay (ELISA). Briefly, the sera (50 μl/well) were coated onto 96-well ELISA plates pre-adsorbed with an anti-cytokine capture antibody overnight at 4°C. The concentrations of antibody for capture of IL-2, IL-4, IL-10, IL-15 and TNF-α were 2.5, 1.0, 1.0, 2.0 and 2.0 μg/ml, respectively. A biotinylated anti-cytokine antibody was then added for an additional 2 h, followed by addition of a 1:1000 dilution of alkaline phosphatase-streptavidin and para-nitrophenyl phosphate (1 mg/ml) in carbonate buffer (pH 9.6). The concentrations of detecting antibodies for IL-2, IL-4, IL-10, IL-15 and TNF-α were 1.25, 1.0, 0.5, 1.0 and 0.5 μg/ml, respectively. Substrate conversion was measured spectrophotometrically at 450 nm. Standard curves were generated with titrated concentrations of recombinant cytokines and were used to quantitate the serum cytokines in pg/ml ranges; the sensitivity of the cytokine assays were: IL-2 > 90 pg/ml, IL-4 > 40 pg/ml, IL-10 > 40 pg/ml, IL-15 > 150 pg/ml and TNF-α > 40 pg/ml.

2.5. Lymphocyte proliferation

PBMC were isolated as previously described [4]. For determination of lymphocyte proliferation in response to the mitogens phytohemagglutinin (PHA), pokeweed mitogen (PWM), phorbol myristate acetate (PMA), and ionomycin, cultures containing 10^5 PBMC (150 μl/well) in 96-well flat-bottom plates were incubated at 37°C for 2 days in the presence of the appropriate reagent(s), followed by the addition of 1 μCi of [^{3}H]-thymidine for an additional 24 h and incorporated radioactivity assessed. PHA was used at concentrations of 10, 5 and 2.5 μg/ml and the maximal value for proliferation used. PWM activity was used in the concentration range of 1.0–0.001 μg/ml in 10-fold dilutions and the maximal value for proliferation used. PMA was used at a concentration of 5×10^{-9} M and ionomycin at a concentration of 5×10^{-7} M. For determination of lymphocyte antiplatelet reactivity in vitro, 7 day APC assays were performed as previously described [14]. In brief, autologous platelets were titrated into cultures containing 2×10^5 PBMC in 96 well round bottom plates and incubated at 37°C for 6 days. The cells were pulsed with 1 μCi of [^{3}H]-thymidine for an additional 24 h and incorporated radioactivity assessed.

2.6. Statistical methods

Regression analysis was used to test the significance of correlations between parameters.

Significant differences between means were determined by the unpaired Student's *t*-test.

3. Results

3.1. Platelets

Patients and healthy controls were categorized into four groups: (a) normals $n=60$, (b) SLE $n=31$, (c) SLE-TP $n=8$, and (d) ITP $n=17$. Due to a limited availability of patient blood, in some cases not all tests were performed on all subjects, therefore the value of "*n*" for any given test may be lower than the total number of subjects for that group.

Seven of the eight SLE subjects classified into the SLE-TP group had platelet counts below $85 \times 10^9\ l^{-1}$. The SLE-TP subject with a platelet count of 142 displayed chronic low grade thrombocytopenia, was positive for platelet-associated IgG (PA-IgG) and had elevated numbers of young platelets (4.9% reticulated platelets vs 1% for control subjects) on the day of analysis. The range and mean whole blood platelet counts for all subjects within each of these groups is shown in

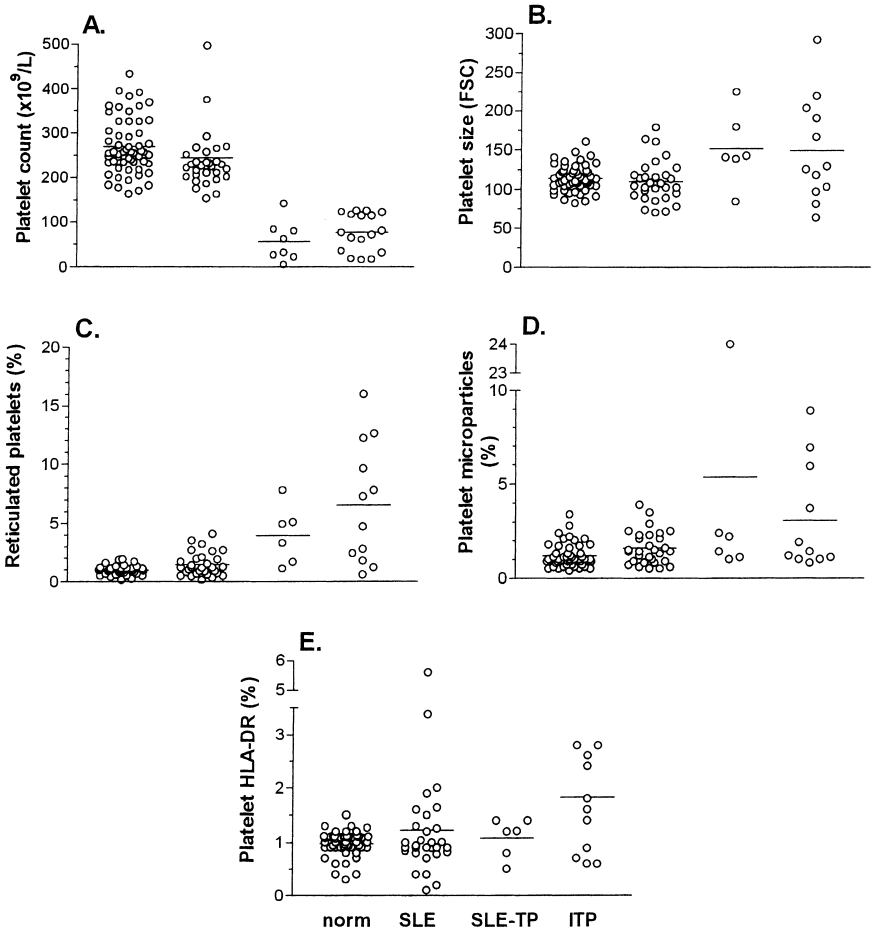

Fig. 1. Analysis of platelets in normal subjects and those with SLE, SLE-TP and ITP. Platelets from individuals grouped as indicated on the *X*-axis in panel E were assessed for five different parameters (panels A–E) as indicated on each corresponding *Y*-axis. Platelet size (panel B) was evaluated by flow cytometry and the mean channel intensity values are displayed. The mean for each group is presented as a horizontal line.

Fig. 1, panel A. The platelets in the SLE-TP and ITP groups were, as expected, larger than in normals or SLE non-thrombocytopenic patients (Fig. 1, panel B), reflecting the higher proportion of young or reticulated platelets in the former groups (Fig. 1, panel C). The SLE-TP and the ITP groups displayed significantly elevated percentages of platelet microparticles, $P < 0.01$ and $P < 0.001$, respectively, compared to normal subjects (Fig. 1, panel D). As shown in Fig. 2, the log proportion of platelet microparticles did correlate inversely with the log platelet count in patients with SLE-TP and ITP ($r^2 = 0.916$); this correlation was not found in SLE patients or healthy controls. Increased platelet associated IgG was observed in 81% of subjects with ITP, all subjects with SLE-TP and 52% of subjects with SLE. We have previously reported that children with ITP can have platelets with small but distinctly elevated levels of HLA-DR and this was confirmed in this study with adults (Fig. 1, panel E). While a few patients with SLE also showed an increase in platelets expressing HLA-DR, this was not seen in the patients with SLE-TP. There was no significant correlation between expression of HLA-DR and platelet count in any group.

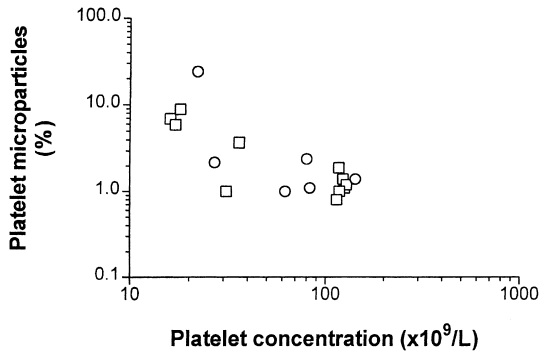

Fig. 2. Comparison between in vivo platelet concentration and microparticle formation in thrombocytopenic subjects. Individuals with ITP (□) and SLE-TP (○) that were assessed for in vivo platelet concentration and microparticle concentration are shown.

3.2. Lymphocyte proliferation

Since, in other autoimmune diseases, abnormalities along the protein kinase C (PKC) pathway may be related to defects in lymphocyte proliferation and to disease onset [15,16], we examined whether pharmacologic activation of PKC by the phorbol ester PMA, in the presence or absence of ionophore or PHA, could cause abnormal lymphocyte proliferation. Proliferation of B and T lymphocytes in response to the B (PWM) and T (PHA) cell mitogens was also measured. As shown in Table 1, patients with SLE-TP displayed significantly more lymphocyte proliferation to PMA than did non-thrombocytopenic SLE patients ($P < 0.001$). Lymphocyte proliferation in response to PHA, PWM, or ionomycin alone or in combination with PMA, was not significantly different in any of the groups.

3.3. Platelet APC assay

A characteristic of ITP is the ability of lymphocytes from some patients to proliferate in

Table 1
Evaluation of in vitro lymphocyte proliferation[a]

Reagent	Normals ($n = 60$)	SLE ($n = 31$)	SLE-TP ($n = 8$)	ITP ($n = 16$)
PHA	$104,600 \pm 6055$	$93,460 \pm 9311$	$110,800 \pm 25,490$	$102,100 \pm 6370$
PWM	$37,640 \pm 2975$	$35,460 \pm 5481$	$37,970 \pm 10,070$	$34,760 \pm 3184$
PMA	$26,240 \pm 6055$	$20,930 \pm 4022$	$70,580 \pm 19,930^{***}$	$32,760 \pm 7279$
Ionomycin	2515 ± 440	3876 ± 1595	5725 ± 2131	5315 ± 1953
PMA + ionomycin	$100,300 \pm 6709$	$89,870 \pm 11,060$	$125,800 \pm 31,330$	$92,710 \pm 9286$
Platelets (APC)	1.60 ± 0.133	$2.48 \pm 0.34^{*}$	1.46 ± 0.54	$2.20 \pm 0.46^{**}$

[a] The results are expressed as the mean cpm for each group ± SEM except for the proliferative response to platelets which is expressed as the mean stimulation index ± SEM.
$^{*}P < 0.01$.
$^{**}P < 0.02$.
$^{***}P < 0.001$.

response to exposure to autologous platelets in an antigen presentation assay [14]. As shown in Table 1, the patients with ITP did demonstrate significantly more proliferation on exposure to platelets than did normal subjects ($P < 0.002$); this was also seen, albeit to a lesser extent, with non-thrombocytopenic patients with SLE ($P < 0.01$), but was not evident with SLE-TP patients.

3.4. Cytokines

The serum levels of the cytokines IL-2, IL-4, IL-10, IL-15 and TNF-α were evaluated (Table 2). Patients with SLE displayed significantly increased levels of IL-10 compared to normals ($P < 0.05$); in contrast, patients with ITP or with SLE-TP did not have increased levels of this Th_2 cytokine. Non-thrombocytopenic patients with SLE also had significantly increased levels of TNF-α ($P < 0.02$); again this was not seen in patients with ITP or SLE-TP. IL-15 was increased in non-thrombocytopenic patients with SLE ($P < 0.01$) and in patients with ITP ($P < 0.05$), but this was not found in patients with SLE-TP.

3.5. Clinical variables

All controls were healthy drug free volunteers with similar age groups and sex distribution as patients. One subject with ITP was on IVIG and another had been taking IVIG and anti-D prior to study and was post-splenectomy; the remainder were untreated. Subjects with SLE and SLE-TP were characterized as having active/inactive disease, presence/absence of anti-phospholipid antibodies (anti-cardiolipin and or lupus anticoagulant), and presence/absence of steroids/Immuran drug therapy. Six subjects with SLE were confirmed to be taking steroids. Of the eight SLE-TP patients, six were taking steroids, one was on Immuran and one was on a combination of steroids and Immuran. Three of these patients were also post splenectomy. Non-thrombocytopenic patients with SLE who had active disease had a significantly increased percentage of platelet microparticles in comparison to those with inactive disease (2.1% vs 1.1%, $P < 0.05$) but less than that observed for SLE-TP subjects (5.3%).

4. Discussion

Thrombocytopenia is a common hematologic complication of SLE and is likely due to antibodies present in SLE patients which react with platelets and cause their clearance. ITP, a diagnosis of exclusion of other causes of thrombocytopenia, is another autoimmune disease where antibodies react with platelets and cause thrombocytopenia. In an attempt to determine which underlying immune parameters are common to SLE, SLE-TP, and ITP, we compared the platelet characteristics and measured cellular immunity in these disorders.

To determine if the thrombocytopenia in SLE is characteristic of antibody-mediated platelet destruction, we evaluated platelet-associated IgG, platelet microparticles, reticulated platelets and platelet size. Both SLE-TP and ITP subjects displayed platelet-associated IgG, and both groups had an increased percentage of platelet microparticles which correlated inversely with in vivo

Table 2
Comparative analysis of cytokine levels from serum[a]

Cytokine	Normals ($n = 60$)	SLE ($n = 31$)	SLE-TP ($n = 8$)	ITP ($n = 16$)
IL-2	1.00 ± 0.09	1.19 ± 0.22	0.86 ± 0.08	0.86 ± 0.06
IL-4	1.00 ± 0.45	1.50 ± 0.41	0.36 ± 0.21	0.54 ± 0.37
IL-10	1.00 ± 0.16	$4.78 \pm 0.39^{*}$	1.03 ± 0.30	1.05 ± 0.31
IL-15	1.00 ± 0.13	$2.36 \pm 0.24^{***}$	1.18 ± 0.37	$2.33 \pm 0.44^{*}$
TNF-α	1.00 ± 0.35	$3.92 \pm 0.33^{**}$	1.84 ± 0.46	2.63 ± 0.58

[a] The mean levels of cytokines is reported as the ratio compared to normal values from 60 control healthy subjects ± SEM.
$^{*}P < 0.05$.
$^{**}P < 0.02$.
$^{***}P < 0.01$ – compared to normals.

platelet number. An increased percentage of reticulated platelets and large platelets as compared to normals or non-thrombocytopenic SLE patients was also found. Collectively, these data are consistent with increased antibody-mediated platelet destruction and increased platelet production in both ITP and SLE-TP.

HLA-DR expression was examined in this study because the direct expression of HLA-DR on platelets has been suggested to play a potential role in ITP [3,4]. In addition, HLA-DR alleles have been shown to either be associated with ITP or be associated with anti-platelet antibodies [17,18]. HLA-DR is the recognition molecule for the T cell receptor of CD4 + Th cells and is the primary initiator of Th cell-dependent immune responses. It is well known that Th cell reactivity with platelets can be observed in ITP [14]. If this reactivity could also be found in SLE-TP, it would suggest that the anti-platelet response in SLE-TP may be due to enhanced anti-platelet T cell help and would suggest a parallel between ITP and SLE-TP. T cell reactivity against platelets was observed in ITP, as expected. Our findings that subjects with SLE-TP do not express increased HLA-DR nor demonstrate platelet-induced T lymphocyte proliferation in the platelet APC assay may indicate that the antiplatelet immune response in these subjects is due to a mechanism independent from direct platelet stimulation of Th cells. This is in contrast to ITP where both increased HLA-DR and Th cell activity directed against platelets were observed, consistent with previous reports [3,4,9]. Significant T cell reactivity against platelets was also observed in SLE, the reason for this T lymphocyte reactivity is unknown at present, but this was not seen at all in SLE-TP. This may indicate that the anti-platelet reactive antibody found in the subjects with SLE-TP may not be due to a direct immune response against platelets but may be due to other immune aberrations associated with SLE.

Analysis of serum cytokines demonstrated significantly increased levels of IL-10, IL-15 and TNF-α in SLE, increased levels of IL-15 in ITP, and no increases in any of these cytokines in SLE-TP. IL-10 and IL-12 are two key regulatory cytokines which have been found to be elevated and suppressed respectively in patients with SLE [19–28] and increased IL-10 levels may relate to disease activity [19,20,26,28]. It was recently reported that the impaired production of IL-12 in patients with SLE could be reversed by neutralizing IL-10 using an anti-IL-10 antibody, implying a regulatory relationship in which increased IL-10 plays a dominant role regulating other cytokine levels and disease activity in SLE [29]. We found no correlation between IL-10 levels and platelet count. The current observation of IL-10 increase in SLE but not in ITP or SLE-TP suggests the thrombocytopenia associated with SLE is induced or regulated by a mechanism more related to ITP than to SLE.

We also observed increased levels of IL-4 in subjects with SLE but decreased levels of IL-4 in subjects with SLE-TP and ITP. Although these IL-4 differences were not statistically significant on their own, reduced levels of PHA-stimulated IL-4 production have been observed by Nugent et al. [30] in childhood ITP. Together, these results are suggestive that subjects with SLE are undergoing Th2 immune stimulation (i.e. increased IL-4 and IL-10) while those with SLE-TP and ITP are not (i.e. decreased IL-4 and normal IL-10) . Since SLE is a systemic autoimmune disease while ITP is an organ-specific disease, these results could be interpreted to suggest that SLE has a Th2-like component [21,28,29] whereas organ-specific (or platelet-specific) autoimmunity has an immune response skewed towards a Th0/Th1-like activation pattern. This is consistent with previous observations [4,5]. The subjects with SLE-TP appeared to have a Th0/Th1-like phenotype similar to primary ITP, and this may be indicative of a changing pathophysiology in those SLE patients who have developed immune thrombocytopenia.

Increased levels of IL-15 were observed in SLE and ITP but surprisingly, not SLE-TP. IL-15 is produced by a wide variety of cells, has IL-2-like properties, and has been demonstrated to enhance the production of TNF-α as well as IL-10 from activated macrophages [31]. It is possible that, in SLE, the increased TNF-α and IL-10 were due to the enhanced levels of IL-15; why IL-15 was not increased in SLE-TP is unexplained, but may be an affect of the small number of subjects.

Lymphocyte proliferative abnormalities have been reported in some subjects with ITP [4,5,8]. Although lymphocyte proliferation in response to PHA and PWM was not significantly different in any of the groups, only subjects with SLE-TP displayed significantly enhanced ($P < 0.001$) proliferation to the PKC activating agent PMA. This enhanced PMA-dependent proliferative effect in subjects with SLE-TP vs those with SLE was unexpected, but aberrations in the PKC-dependent pathway have been reported by us in a murine model of autoimmunity [15,16] and this may indicate that defects in PKC-dependent proliferation may be associated with autoimmunity.

The above tests were related to clinical variables in the group with SLE. Although some of these variables were influenced by clinical condition (i.e. increased platelet microparticles in SLE subjects with active disease), the presence or absence of active disease or drug therapy did not appear to influence or explain the significant differences between the groups as discussed above.

In summary, we found that although similar at the platelet level (platelet-associated IgG, microparticles, platelet size and reticulated platelets), differences exist at the cellular immune level between SLE and ITP and SLE-TP. Importantly, the increase in IL-10 which is said to be regulatory in disease activity in SLE was not seen in ITP or SLE-TP. While patients with SLE showed a cytokine pattern suggestive of a Th2 response, patients with ITP and SLE-TP showed similar patterns of IL-4 and IL-10 secretion skewed to a Th0/Th1 response. Other aspects of cellular immune function, however, showed differences between SLE-TP and ITP, e.g. platelet HLA-DR expression, T cell proliferative response to platelet exposure and to PMA and IL-15 secretion, suggesting that the immune thrombocytopenia in SLE patients is not a 'true ITP', but may be modified by immune abnormalities integral to SLE.

Acknowledgements

This work was supported by a grant from Cara Operations Ltd.We thank Margaret Kardish, and Wilda Chang for their technical expertise in flow cytometric analysis and Anna Porretta for her help with the proliferation assays. We extend a special thanks to Ms. Sharon McFadden from Cara Food Operations Ltd. for her organizational contribution. We further thank the patients who participated in this study and Drs. Shupak, Gershon, and Perlin of St Michael's Hospital for their help and support of this work. AHL is a Bayer/CRCS/MRC Scholar.

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