Depressed Functional and Phenotypic Properties of T but not B Lymphocytes in Idiopathic Thrombocytopenic Purpura

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Chronic idiopathic thrombocytopenic purpura (ITP) is an autoimmune disorder in which the abnormality in cellular immunity has remained only vaguely defined. Previously we have shown that patients with ITP in its active phase have abnormal T cell subsets. We then examined the phenotypes of T and B lymphocytes in an additional 28 patients with ITP and 32 age- and sex-matched normal controls and compared the lymphocytes’ capacity to respond to polyclonal T, T cell-dependent B, and B cell mitogens. Blastogenesis to optimal (5.0 μg/mL) and suboptimal (0.5 μg/mL) concentrations of the polyclonal T cell mitogens were markedly depressed in patients compared with normal controls (P < 0.0005). Similarly, a severe depression in response was noted with the polyclonal T cell–dependent B cell mitogen (P < .000001). No difference was seen, however, with the polyclonal B cell mitogen. The proportions of pan-T and T helper/inducer lymphocytes were significantly depressed (P < .005 and P < .000006 respectively), and the T suppressor/cytotoxic lymphocytes increased (P < .02) in patients relative to controls. But there was no difference in the proportion of B lymphocytes or in their functional response. The abnormal cellular immunity appears to be due to a defect in the T lymphocyte population without involvement of the B lymphocytes.

Patient population. Twenty-eight adults (21 women, seven men) with ITP of greater than 6 months’ duration were studied. The diagnosis of ITP was based on symptomatic thrombocytopenia with megakaryocytosis, without any clinical or laboratory evidence of an underlying multisystem disorder. All patients were negative for connective tissue disease markers, human immunodeficiency virus antibodies, other autoimmune disorders, or evidence of malignancy. Their platelet counts were less than 100,000/μL (mean ± SEM, 33,890 ± 4,340). Controls were normal healthy volunteers (24 women, eight men of similar age and race) with counts of greater than 200,000/μL.

Therapy. All patients (with three exceptions) were not receiving any drug for at least a month before the study. This length of time ensured that drug-induced changes in the immune system were no longer present. Of the three individuals receiving drugs one was receiving conjugated estrogens, 0.3 mg, and two were receiving prednisone, 5.0 mg daily. In the control group, all were healthy and receiving no medications known to have immunomodulatory effects.

Mononuclear cell preparation. Venous blood (20 mL) was collected in preservative-free ammonium heparin (Monoject Scientific, St Louis) and processed aseptically. Their peripheral blood mononuclear cells (PBMCs) were isolated by sodium diatrizoate–Ficoll density gradient centrifugation. The PBMCs were washed three times with Hanks’ balanced salt solution and resuspended to a concentration of 2 × 10^6 cells/mL, with culture medium consisting of RPMI 1640 supplemented with 5% fetal bovine serum and 100 U/mL each of penicillin and streptomycin. Cell viability was checked by trypan blue dye exclusion and always found to be greater than 90%.

Mitogens and sera. Lyophilized concanavalin A (Con A) (Behring Diagnostics, La Jolla, CA), phytohemagglutnin (PHA), and pokeweed mitogen (PWM) (Sigma Chemical Co, St Louis) were dissolved in RPMI 1640 to a concentration of 100 μg/mL; 1-mL portions were stored at −20°C until used. *Staphylococcus aureus* Cowan 1 (Staph A) (Bethesda Research Laboratories, Gaithersburg, MD) was obtained fixed in formalin. They were centrifuged at 1,000 g for ten minutes to discard the supernatant, washed twice in RPMI 1640, and resuspended to a concentration of 10^5 cells/mL; this was followed by storage at 4°C in 0.5-mL portions. Fetal bovine serum (GIBCO, Chagrin Falls, OH) that had been screened for...
mycoplasma and virus was heated at 56°C for 30 minutes. Aliquots of 30 mL were frozen at −20°C until required. All mitogens and fetal bovine sera were added to the cultures from this same lot.

**Culture of PBMCs.** PBMCs (1 × 10^6) were placed in 96-well, flat-bottomed microtiter plates (Becton Dickinson, Oxnard, CA). PHA and Con A were added in quadruplicates at four concentrations (5.0, 2.5, 0.5, and 0.25 µg/mL) PWM at seven concentrations (5.0, 0.5, 0.25, 0.125, 0.063, 0.031, and 0.016 µg/mL) and Staph A at 10^4, 10^5, and 10^6 cells/mL. The ranges of concentration for each mitogen were derived from preliminary experiments on optimal and suboptimal blastogenic responses in patients and controls. After addition of the mitogens, the culture medium was added to each well to bring the volume up to 0.25 mL. The plates were covered and cultured at 37°C in a 5% CO₂ humidified air incubator for four days. At 91 hours 0.5 µCi of [methyl-3H]-thymidine at 6.7 Ci/mmol (New England Nuclear, Boston) was added to each well and further incubated for five hours. At 96 hours the plates were removed, the PBMCs lysed with water, and the lysates incubated for five hours. At 96 hours the plates were removed, the PBMCs rinsed with PBS, and the washing procedure was repeated twice. Fluorescein-conjugated goat anti-mouse IgG was used. The percentage of nonspecific fluorescence was consistently less than 2.0. Monocyte contamination of the lymphocyte populations identified and gated by forward and right-angle light scattering measurements was less than 2.0% with the Mo2 antibody (Coulter Immunology). The interday coefficient of variation of the counts due to nonspecific blastogenesis in culture medium alone. Results were expressed as cpm/10^6 cells. The interday coefficient of variation of blastogenesis in the same healthy individual and patient was less than 10% and 15%, respectively. The cell viability at 96 hours was consistently greater than 90%.

**Lymphocyte phenotypes.** The remainder of the PBMCs were used for phenotyping lymphocytes and subsets by using the procedure as described by us previously15,17 with two minor changes. The Leu series monoclonal antibodies were replaced by the Coulter clones T3 (pan-T), T4 (T helper/inducer [T₄]), T8 (T suppressor/cytotoxic [T₈]), and B4 (pan-B) (Coulter Immunology, Hialeah, FL). The indirect immunofluorescence staining procedure was adopted to achieve better resolution of the T8 and B4 markers by increasing the signal-to-noise ratio. The PBMCs (0.5 mL) were transferred to a series of tubes and centrifuged at 375 g for ten minutes to pellet them.

**Indirect immunofluorescence staining.** Monoclonal antibody (5 µL) was added to each PBMC (10^6) pellet, gently resuspended and incubated at 4°C for 40 minutes. One milliliter of phosphate-buffered saline (PBS) was then added, the mixture centrifuged at 375 g for ten minutes, and the supernatant discarded. The washing procedure was repeated twice. Fluorescein-conjugated goat antimouse IgG (5 µL) was added to each PMBC pellet and further incubated at 4°C for 40 minutes. The procedure was repeated twice. The PBMC was finally resuspended in 0.9 mL of PBS; this was followed by the addition of 0.1 mL of 20% paraformaldehyde and stored at 4°C until measured by flow cytometry on an EPICS V (Coulter Electronics, Hialeah, FL). For nonspecific background staining, mouse IgG was used. The percentage of nonspecific fluorescence was consistently less than 2.0. Monocyte contamination of the lymphocyte populations identified and gated by forward and right-angle light scattering measurements was less than 2.0% with the Mo2 antibody (Coulter Immunology). The interday coefficient of variation of the percentage of fluorescence in the same healthy individual and patient was less than 9% and 11%, respectively.

**Statistics.** To analyze the results, the two-sample Student's t test and Pearson's correlation analysis were used. In using the two-sample t test, the SDs of the samples were tested for nonhomogeneity. If there was a significant difference, the unpoled variance t test was used.

**RESULTS**

**Lymphocyte blastogenesis to mitogens.** The blastogenic response of lymphocytes to optimal concentrations (5.0 µg/mL) of PHA and Con A was markedly depressed in patients with ITP in its active phase compared with normal controls (Fig 1). The severity of the depression was greater with Con A (P < .000001) than PHA (P < .000005). This pattern of depressed blastogenesis was also seen with suboptimal concentrations (0.5 µg/mL) of the two mitogens (Table 1). Similarly, a marked decrease in blastogenic response (P < .000001) was also observed with an optimal concentration (0.125 µg/mL) of PWM (Fig 1). In contrast, no difference in response between patients and normal controls was noted with Staph A (Fig 1). Preincubation of randomly selected PBMCs of patients for one hour at 37°C followed by washing and resuspension to the same cell concentration showed no change in the blastogenic response relative to their counterparts that did not undergo such a procedure (data not shown). No correlation was found to exist between the blastogenic response of patients and their platelet counts and duration of the disease; however, in patients and normal controls the blastogenic responses to optimum concentrations of PHA and Con A were inversely proportional to their ages (Figs 2 and 3).

**T cell subsets and B cells.** The proportions of pan-T lymphocytes and the T₈ subset were significantly depressed and the T₆₅ subset increased in patients compared with normal controls, but there was no difference in the proportion of pan-B lymphocytes (Fig 4). Consequently, the T₈/T₆₅ ratios were depressed (P < .005). Owing to a greater decrease in the proportion of the patients' T₈ subset (P < .000005) to that of pan-T lymphocytes (P < .005)

![Fig 1. Blastogenic responses (cpm/10^6 cells) to optimal mitogen concentrations in normal controls (○) and patients with active ITP (●)]. Optimal concentrations of PHA and Con A were 5 µg/mL; PWM, 0.125 µg/mL; and Staph A, 10^6 cells/mL. The boxed areas represent the mean ± 1 SD. The bracketed numbers (') are the P values.
when compared with normal controls, only the number of Th\textsubscript{1} lymphocytes were significantly depressed ($P < .001$) in patients (Table 2). In patients there were no correlations between the proportions and numbers of pan T, T cell subsets, and B lymphocytes with age, platelet counts, and duration of disease. In normal controls, however, the proportions of the Th\textsubscript{1} lymphocytes were directly related ($r = .46$, $P < .01$) and Th\textsubscript{2} inversely correlated ($r = .43$, $P < .02$) with their age (Fig 5).

**T cell subsets and lymphocyte blastogenesis.** Because there were depressed proportions of pan-T and Th\textsubscript{1} lymphocytes in patients relative to normal controls, comparisons were made between the proportions and numbers of lymphocytes with their blastogenesis. In patients and normal controls, no statistically significant correlations were found between the proportions and numbers of pan-T and T cell subsets with the blastogenic response to PHA, Con A, and PWM. In normal controls, the proportion and number of B lymphocytes were directly correlated with the Staph A response (Fig 6) but, however, not in the case of the patient population.

**Table 1. Lymphocyte Blastogenesis to Suboptimum (0.5 g/mL) Concentrations of PHA and Con A in Patients and Normal Controls**

<table>
<thead>
<tr>
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<th>PHA</th>
<th>Con A</th>
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<tr>
<td>Patients ($n = 28$)</td>
<td>16,034 ± 17,671</td>
<td>5,119 ± 4,942</td>
</tr>
<tr>
<td>Normal controls ($n = 32$)</td>
<td>42,704 ± 27,569</td>
<td>22,566 ± 23,876</td>
</tr>
<tr>
<td>$P$ Value</td>
<td>&lt;.00005</td>
<td>&lt;.0005</td>
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Results are ± 1 SD.

Fig 2. Blastogenic responses (cpm/10\textsuperscript{5} cells) to optimal PHA (●) and Con A (○) concentrations and their relation to age in patients with active ITP. The continuous line (—-) relates PHA and the broken line (---) relates Con A with age.

**DISCUSSION**

Our results show that the functional abnormality in the lymphocytes of patients with ITP in its active phase primarily resides in the T lymphocytes without any apparent involvement of the B lymphocytes. The depressed blastogenic response to PHA, Con A, and PWM were not dependent on the concentrations of the mitogens used because they were
In contrast, the B cell cell-dependent mitogenic stimuli. decreased at optimum and suboptimum concentrations, thereby indicating a uniformly unresponsive state to T and T cell-dependent mitogenic stimuli. In contrast, the B cell response to Staph A, a polyclonal B cell mitogen, was not depressed. Our findings on depressed blastogenesis to PHA and Con A differs with depressed. Our findings on depressed blastogenesis to PHA and Con A. Similarly, they too decreased the blastogenic response. They were unable to reproduce either the active disease. They were unable to reproduce either the impaired blastogenesis with PHA and Con A from patients with ITP showed impaired blastogenesis with PHA and Con A but not when separated and washed. They attributed the correction of the impairment to removal of blocking factors or antibodies. We have compared the blastogenic response of lymphocytes of randomly selected patients that were preincubated for one hour at 37°C to remove any bound antibodies with those that were not and found no difference in their response. This lack of interference by antibodies was also shown by Kaufman et al who found that plasma from ITP patients on incubation with normal control lymphocytes tended to increase rather than decrease the blastogenic response. Similarly, they too found diminished blastogenesis to PHA and Con A. Waldschmidt and Mueller-Eckhardt have shown that the response to PHA is again significantly reduced in ITP patients with active disease. They were unable to reproduce either the proliferative or inhibitory effects of platelet-antibody complexes on lymphocytes as shown by others.

The main difficulty in interpreting the depressed blastogenesis to PHA and Con A is the functional capability of monocytes to help T lymphocytes in PHA- and Con A-induced blastogenesis. Recently it was shown with reconstitutive experiments that the depressed PHA response in ITP resides in T lymphocytes and not monocytes. Further, this group has found impaired T lymphocyte mitogenesis to the OKT3 monoclonal antibody in ITP. Although we did not use OKT3 in the functional studies, our phenotypic analysis of lymphocytes showed a decreased proportion of pan-T (OKT3) in patients relative to normal controls in the present and previous study. Some investigators claim that the T3 complex is the site of attachment of PHA and the amount of surface T3 is a critical variable in PHA activation. Others, by more sophisticated and elaborate techniques, suggest that PHA may trigger T lymphocytes into blastogenesis by partially interacting with carbohydrate moieties of the T, that is the T cell antigen receptor. The lower percentage of the T3 receptors may prevent complete transfer of the mitogenic signal induced by either PHA or T3 antibody. Owing to the depressed proportion of the pan-T lymphocytes in patients, the proportion and number of the Th subset was also markedly decreased. It has been shown that the Th subset is more responsive to PHA and Con A and may be a further reason for the depressed blastogenesis.

Although the responses of the patients' lymphocytes were depressed to PHA, Con A, and PWM and the proportions of pan-T and Th were decreased and Th increased with respect

<table>
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<tr>
<th>Patients (n = 28)</th>
<th>Normal controls (n = 32)</th>
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<tbody>
<tr>
<td>Pan-T</td>
<td>1,744 ± 1,390</td>
</tr>
<tr>
<td>Th</td>
<td>905 ± 352</td>
</tr>
<tr>
<td>Th</td>
<td>885 ± 1,170</td>
</tr>
<tr>
<td>B</td>
<td>167 ± 160</td>
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<tr>
<td>P value</td>
<td>NS</td>
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Results are ± 1 SD.

Fig 5. Proportions of Th (D) and Th (O) lymphocytes and their correlation with age in normal controls. The continuous line (-----) represents Th and the broken line (-----) represents Th with age.

Fig 6. Proportion (D) and number (O) of pan-B lymphocytes and their correlation with blastogenic response (cpm/10^6 cells). The continuous line (-----) denotes the proportion and the broken line (-----), numbers.
LYMPHOCYTE FUNCTION AND PHENOTYPE IN ITP

mitogenic lectin from *Phaseolus vulgaris* does not recognize the T3 antigen of human T lymphocytes. Eur J Immunol 15:479, 1985


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