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

By Ravinda Mylvaganam, Rolando O. Garcia, Yeon S. Ahn, Philippa G. Sprinz, Chae I. Kim, and William J. Harrington

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 (6.0 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 < .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.

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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 added to the cultures were 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 10^6, 10^7, and 10^8 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 deposited on a filtermat with a semiautomatic cell harvester (Skatron, Inc, Sterling, VA). The disks of filter paper were transferred to minivials, followed by the addition of 1 mL of scintillation fluid and measurement in a Beckman LS 7000 scintillation counter (Beckman Instruments, Inc, Fullerton, CA). The blastogenic response was measured as the uptake of [methyl-3H]-thymidine in counts per minute after subtraction of the counts due to nonspecific blastogenesis in culture medium alone. Results were expressed as cpm/10⁶ 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 previously 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 (10⁶) 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 PBMC 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 flowcrometry 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 T₄ 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/T₄ ratios were depressed (P < .005). Owing to a greater decrease in the proportion of the patients' T₄ subset (P < .000005) to that of pan-T lymphocytes (P < .005)
LYMPHOCYTE FUNCTION AND PHENOTYPE IN ITP

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

<table>
<thead>
<tr>
<th></th>
<th>PHA</th>
<th>Con A</th>
</tr>
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<tbody>
<tr>
<td>Patients (n = 28)</td>
<td>16,034 ± 17,761</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>
</tr>
</tbody>
</table>

Results are ± 1 SD.

when compared with normal controls, only the number of ThI 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 ThI lymphocytes were directly related ($r = .46$, $P < .01$) and Tc, 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 ThI 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.

Fig 2. Blastogenic responses (cpm/10⁵ 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-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 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 with ITP showed impaired blastogenesis with PHA and Con A. Waldschmidt and Mueller-Eckhardt have shown that the response to OKT3 monoclonal antibody in ITP. Although we have found impaired T lymphocyte mitogenesis to the OKT3 receptors may prevent complete transfer of the mitogenic signal induced by either PHA or T3 antibody. The lower percentage of pan-T lymphocytes and their correlation with blastogenic response (cpm/10^5 cells).

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 T3 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 Tn subset was also markedly depressed. 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 Tn increased with respect...
to normal controls, there was found to be neither a linear nor
a nonlinear association between function and phenotype.
This indicates that the blastogenic response is dependent on
other intermediate steps that may be defective; for example,
the expression and secretion of interleukin-2 as reported in
systemic lupus erythematosus or the correlation may lie
with the subpopulations of T_h and T_s lymphocytes such as
the T inducer of suppressors (T4 + 2H4 +) and T suppressor
lymphocytes (T8 + Leu15 +). Work is in progress to measure
and compare the subpopulations with blastogenesis by dual-
color immunofluorescence flow cytometry. Another plausible
explanation for the lack of correlation between function and
phenotype in patients is the presence of an association
between age and T cell subsets or blastogenic response. In
normal controls, age is directly related to the proportion
and number of T_s and inversely to the proportion of T_h lympho-
cytes, whereas this is not the case in the patient population.
In both patients and normal controls, however, the age
negatively correlates with the PHA and Con A response.
This dichotomy in relationship in normal controls between
age tending to have higher T_s but depressed rather
than enhanced mitogenic response and the lack of such an
association in patients tends to reduce the possibility of any
correlations. In contrast to the depressed blastogenic response
of T lymphocytes and decreased proportions of
pan-T and T_h in patients, however, functional properties and
phenotypic characteristic of the B lymphocytes were no
different from those of the normal controls, which suggests
that the cellular defect in ITP is mainly confined to the T
lymphocyte and its dependent functions. That there is a
direct relationship between the proportion and number of B
lymphocytes with their Staph A response in normal controls
but not in patients implies subtle differences not revealed by
our present studies. It has been shown that B1 + B2 + and
B1 + B2 - are distinct B lymphocyte subsets, with the former
requiring both T lymphocytes and PWM to produce antibod-
ies whereas the latter needs only T lymphocytes. B1 and B2
monoclonal antibodies may be useful in analyzing propor-
tions of the two B cell subsets in ITP and revealing any
abnormalities. By using soluble proteins such as tetanus
toxoid, it can be further determined whether the abnormality
of the T lymphocyte is an inability to recognize and be
triggered by a specific stimulus from a recall antigen or an
inability to mount an effector function after cell triggering.77
Studies of this nature are underway, and we have shown that
patients with ITP in its active phase appear to have abnormal
T cell immunity that is not due to plasma factor(s) but rather
to an intrinsic defect in the T lymphocytes.

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