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

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Chronic idiopathic thrombocytopenic purpura (ITP), a well-defined autoimmune hematologic disorder, occurs more commonly in women than men with a ratio of 2:1 to 3:1. In this disorder, platelets are sensitized with an IgG autoantibody and rendered susceptible to phagocytosis by macrophages of the mononuclear phagocyte system. The existence of a humoral factor in ITP was well established 3 decades ago and was subsequently followed by the development of different methods to measure platelet autoantibodies for use as a laboratory test to help in the diagnosis of ITP. Although platelet autoantibody measurements are routinely available in most special hematology centers, they have no clear value in treatment and prognosis.

We have used monoclonal antibodies against T and B lymphocytes to monitor the cellular immune status of patients with ITP. Patients with ITP in its active phase had abnormal T cell subsets, with the abnormality being more severe in women than men, and therapy, particularly with danazol, can reverse this abnormality. Accordingly, we compared the functional properties of lymphocytes of patients with ITP in its active phase with those of sex- and age-matched normal controls. Patients with ITP in its active phase appear to have a depressed lymphocyte response to polyclonal T and T cell–dependent B cell mitogens but not to polyclonal B cell mitogen. This paper presents these data and suggests their clinical utility.

MATERIALS AND METHODS

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 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), phytohemagglutinin (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 10 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 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, 10^2, and 10^3 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/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 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 µL) 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 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 flowcytometry 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 ratio was 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)
when compared with normal controls, only the number of Th 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 lymphocytes were directly related (r = .46, P < .01) and Tc inversely correlated (r = .43, P < .02) with their age (Fig 3).

**T cell subsets and lymphocyte blastogenesis.** Because there were depressed proportions of pan-T and Th 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.

**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...
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 differ with previous study. Some investigators claim that the T3 receptors may prevent complete transfer of the mitogenic signal induced by either PHA or T3 antibody.21 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 complex on lymphocytes.22 The lower percentage of surface T3 is a critical variable in PHA activation.23 Others, in the functional studies, our phenotypic analysis of 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 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 Th1 subset was also markedly depressed. It has been shown that the Th1 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 Th1 were decreased and Th2 increased with respect to normal controls, this is not evident in 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 al24 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-Eckhardt25 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.17-19

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.26 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 the T3 receptors may prevent complete transfer of the mitogenic signal induced by either PHA or T3 antibody. The lower percentage of 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 Th1 subset was also markedly depressed. It has been shown that the Th1 subset is more responsive to PHA and Con A and may be a further reason for the depressed blastogenesis.

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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 $T_n$ and $T_s$ lymphocytes such as the T inducer of suppressors ($T_4 + 2H4 +$) and T suppressor lymphocytes ($T_8 + $Leu $15 +$). Work is in progress to measure and compare the subpopulations with blastogenesis by dual-color immunofluorescence flowcytometry. 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 $T_n$ and inversely to the proportion of $T_s$ lymphocytes, 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 older 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 $T_n$ 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 $B_1 + B_2 +$ and $B_1 + B_2 -$ are distinct B lymphocyte subsets, with the former requiring both T lymphocytes and PWM to produce antibodies whereas the latter needs only T lymphocytes. B1 and B2 monoclonal antibodies may be useful in analyzing proportions 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. 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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