Leukocyte Migration Inhibition of Buffy Coats From Patients With Autoimmune Thrombocytopenic Purpura When Exposed to Normal Platelets: Modulation by Transfer Factor

By W. Borkowsky and S. Karpatkin

Cellular-mediated immunity was studied in autoimmune thrombocytopenic purpura (ATP) patients by investigating leukocyte migration inhibition (LMI) following the interaction of normal platelets with patients' lymphocytes. When normal platelets were incubated with leukocyte buffy coats of ATP patients, the migration index (MI) was significantly impaired compared to buffy coats from normal subjects, employing 4 different concentrations of platelets. At the highest platelet concentration (10⁷/ml), MI was 0.87 ± 0.04 (SEM) for ATP lymphocytes compared to 1.05 ± 0.06 (p < 0.01) for normal lymphocytes. Nine of 21 patients had an MI < 0.80, whereas all control subjects had MIs > 0.85. Similar results were obtained at 2 different platelet membrane concentrations. At 500 µg/ml, the MI for ATP lymphocytes was 0.74 ± 0.04, compared to 0.98 ± 0.06 (p < 0.01) for normal lymphocytes (12 experiments). An inverse relationship was noted between platelet count and lymphokine production in ATP patients (r = 0.815, p < 0.001, 10 experiments). Transfer factor from an ATP patient in remission converted an abnormal LMI response of 0.68 ± 0.04 from a patient with severe thrombocytopenia to 0.84 ± 0.07 (p < 0.005, 8 experiments). Similar results were obtained with transfer factor from 2 other patients in remission. Transfer factor from a patient with severe thrombocytopenia converted a normal response of 1.04 ± 0.06 of normal subjects to a lower response of 0.88 ± 0.04 (p < 0.03, 12 experiments). Thus, lymphocytes of ATP patients are primed to recognize and be perturbed by normal platelets, whereas normal lymphocytes are not. This indicates specificity of the antigen-lymphocyte reaction in ATP patients. Transfer factor is capable of modulating this response in vitro.

Autoimmune thrombocytopenic purpura (ATP) is a hemostatic clinical disorder mediated by the presence of a humoral antiplatelet antibody. In 1970, Piessens et al. first suggested a role for cellular-mediated immunity in the pathogenesis of this disorder by demonstrating increased lymphocyte transformation of ATP patients' lymphocytes by platelets. Other investigators confirmed these observations. Similar observations were made by Clancy, who also noted leukocyte migration inhibition (LMI) by autologous platelets in 9 of 10 patients tested, and by Morimoto et al., who noted LMI in 6 of 14 patients tested. This technique measures lymphokine release by "educated" lymphocytes exposed to the antigen (platelets) to which they have been sensitized. Unfortunately, except for the study of Morimoto et al., who also studied homologous platelets, the antigens used in these studies were autologous platelets, which are now known to be coated with antiplatelet antibody. Since antigen-antibody complexes can both stimulate lymphocyte transformation and inhibit leukocyte migration, it is possible that nonspecific lymphocyte stimulation was observed in these studies. When homologous platelets were employed, LMI was seen in only 2 of 14 patients. Since this study employed an indirect assay, in which lymphocytes were cultured for 3 days with platelets, it is possible that antiplatelet antibody production could have occurred and that consequent platelet–antiplatelet antibody complexes may have stimulated lymphocyte release of LMI factor.

The purpose of the present investigation was to determine whether cellular-mediated immunity plays a role in the pathogenesis of ATP. An LMI assay was therefore designed to test whether normal (allogeneic) platelets or their solubilized membranes were capable of stimulating lymphocytes of ATP patients to release LMI factor.

In this article, we present evidence for cellular-mediated immunity of ATP patients that is directed against platelets of normal individuals. Cellular-mediated immunity was inversely related to the patients' peripheral blood platelet count. This cellular-mediated immunity was inhibited (reregulated) by transfer factor [dialyzable leukocyte extract (DLE)] obtained from ATP patients in remission. In addition, normal human lymphocytes could be "educated" to produce cellular-mediated immunity against allogeneic platelets by incubation with DLE obtained from an ATP patient with severe thrombocytopenia. Thus, ATP patients have cellular-mediated immunity that is directed against platelets and modulated by DLE.

MATERIALS AND METHODS

Leukocyte Donors

Heparinized peripheral blood was obtained from normal healthy volunteers and from individuals with classic chronic autoimmune thrombocytopenic purpura for both the LMI assay and the produc-
tion of dialyzable leukocyte extract (DLE). Patients had been ingesting 0–10 mg prednisone/day at the time of testing.

**Preparation of DLE**

Heparinized peripheral blood was supplemented with dextran (Sigma Chemical Co., St. Louis, MO) to a final concentration of 0.6%. The blood was sedimented at room temperature at 1 g, and the supernatant collected and centrifuged at 160 g for 10 min at room temperature to collect leukocytes. The leukocyte pellet was resuspended in Minimal Essential Medium (MEM, GIBCO, Grand Island, NY), washed twice in 0.01 M phosphate-buffered saline (PBS), pH 7.4, and resuspended in sterile distilled water to a concentration of 5 × 10⁶ mononuclear cells/ml. This material was subjected to 10 freeze–thaw procedures, followed by dialysis under vacuum, with dialysis tubing having a 12,000 mol wt exclusion size (Thomas Scientific Apparatus, Philadelphia, PA). The dialysate was stored at 4°C for more than 2 wk until use in the LMI assay.

**Direct LMI Assay**

Peripheral blood from individuals with ATP or from normal healthy volunteers was sedimented with dextran as directed above. The leukocyte pellet was washed 3 times in Medium 199 (GIBCO) and dispensed into 12 x 75 mm plastic tubes (Falcon 2058, Oxnard, CA) at a concentration of 7 x 10⁶ cells/ml. The tubes were incubated for 30 min at 37°C and washed once with 5 ml of Medium 199. The tubes were centrifuged at 160 g for 10 min at 20°C and the supernatant completely removed. To the cell pellet was added 30 μl of Medium 199 and 3 μl of DLE. The tubes were incubated at 37°C in a 5% CO2 atmosphere, and the migration of 900 μl/ml (Massachusetts Dept. of Public Health, Boston, MA), or tetanus toxoid, at a concentration of 6–10 mg/ml. With a 2-mm diameter hole puncher.

**Preparation of Agarose Plates**

The plates were prepared by dissolving 2 g agarose (BioRad, Richmond, CA) in 100 ml of sterile distilled water. To this were added 57 ml of distilled water, 18 ml of 10x Medium 199 concentrate, 20 ml of horse serum, and 5 ml of a 7.5% solution of NaHCO₃. Eight-milliliter aliquots were added to plastic culture dishes (Falcon, 1055 Y Petri dish). When the agarose had hardened, wells were cut between the mean and standard deviation. Using this assay, expression of cell-mediated immunity by DLE can also be observed using this assay.

**Preparation of Whole Platelet and Platelet Membrane Suspensions**

Platelets were prepared from platelet-rich plasma obtained from the New York Blood Center and suspended in a bicarbonate-buffered human Ringer solution, pH 7.1, as described previously. Platelet membranes were prepared from platelets washed in the above buffer by freezing and thawing, sonicating, and centrifuging over a 30% sucrose cushion, as described previously. The platelet membranes were then suspended in human Ringer solution or saline (rather than solubilizing in 1% Triton X100) at a protein concentration of 6–10 mg/ml.

**RESULTS**

**Immune Recognition of Homologous Platelets**

Leukocytes from normal individuals or from individuals with active autoimmune thrombocytopenia were incubated with washed homologous platelets and placed into agarose wells overnight. The release of the lymphokine leukocyte inhibition factor (LIF) was measured by calculating the migration index (MI) for each population of leukocytes at a given concentration of platelets.

Our results, determined from assays performed on quadruplicate samples from 4 different platelet concentrations, are shown in Fig. 1. A statistically significant difference in MI of patients versus simultaneously run control subjects was noted at each concentration of platelets employed (paired Student’s t test). At the highest platelet concentration tested (10⁸ platelets), the mean MI was 0.87 ± 0.04 (SEM) for ATP lymphocytes, compared to 1.05 ± 0.05 (p < 0.01) for normal individuals.

![Fig. 1. Effect of homologous platelets obtained from normal subjects on the migration index of leukocytes obtained from the Buffy coat of ATP patients (O) as well as allogeneic normal subjects (O). Horizontal line refers to the mean.](https://example.com/fig1.png)
from ATP patients as well as normal subjects and the migration of normal leukocytes at both platelet concentrations. As can be seen from Table 1, the MI of ATP patients' leukocytes was significantly lower than that of normal leukocytes at both platelet concentrations used (500 and 250 μg/ml), with a mean MI of 0.74 ± 0.04 and 0.86 ± 0.04, respectively, for ATP leukocytes (20 individuals) and a mean MI of 0.98 ± 0.08 and 1.07 ± 0.08, respectively, for normal leukocytes (12 individuals). These findings were significant at the p < 0.01 and p < 0.03 level, respectively (paired Student's t test).

Immune Recognition of Platelet Membranes

The assay described above was repeated using a platelet membrane suspension instead of intact platelets. As can be seen from Table 1, the MI of ATP patients' leukocytes was significantly lower than that of normal leukocytes at both platelet concentrations used (500 and 250 μg/ml), with a mean MI of 0.74 ± 0.04 and 0.86 ± 0.04, respectively, for ATP leukocytes (20 individuals) and a mean MI of 0.98 ± 0.08 and 1.07 ± 0.08, respectively, for normal leukocytes (12 individuals). These findings were significant at the p < 0.01 and p < 0.03 level, respectively (paired Student's t test).

Correlation of MI With Peripheral Blood Platelet Count

Figure 2 demonstrates an inverse correlation between the MI induced by homologous platelet membranes and the patient's peripheral blood platelet count \( (r = -0.815, p < 0.001, n = 10) \).

Lack of Immune Recognition of Other Antigens

The effect of tetanus toxoid (90 LF units/ml) and keyhole limpet hemocyanin (100 μg/ml) antigens on the MI of several ATP patients or normal subjects was also measured. Significant differences were not seen in the response to these antigens by these two groups.

Effect of Dialyzable Leukocyte Extracts From a Patient With ATP in Remission on the MI of ATP Leukocytes Reacting to Platelet Membrane Antigens

DLE prepared from leukocytes of normal individuals or an individual with a diagnosis of ATP in remission was used to modify the responses of ATP leukocytes to platelet membranes in the LMI assay. The patient in remission is a 52-yr-old white female with intermittent chronic ATP of 6 yr duration. The addition of her leukocyte dialysate significantly increased the MI of ATP-derived leukocytes reacting to platelet membranes at concentrations of 500 and 250 μg/ml (Fig. 3). Thus, DLE treatment of ATP leukocytes increased the mean MI from 0.68 ± 0.04 to 0.84 ± 0.07 at a platelet membrane concentration of 500 μg/ml \( (p < 0.005, \text{panel } 3, 8 \text{ experiments}) \), while it increased the mean MI from 0.92 ± 0.04 to 1.05 ± 0.05 at a platelet membrane concentration of 250 μg/ml \( (p < 0.03, \text{panel } 4) \). Similar results were obtained with a 46-yr-old white female in remission for 6 yr whose DLE increased the mean MI of leukocytes of 6 ATP patients from 0.75 ± 0.05 to 0.89 ± 0.06 at high concentration of antigen and increased the mean

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<th>Table 1. Mean Migration Index for Homologous Platelet Membranes Reacting With ATP Patients' Leukocytes*</th>
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*Numbers refer to mean ± SEM.

Fig. 2. Correlation of the migration index with the peripheral blood platelet count of ATP patients. Normal platelet membranes, 500 μg/ml, were incubated withuffy coat leukocytes obtained from ATP patients as well as normal subjects and the migration indices obtained. The ratio of control MI/patient correlated with the peripheral blood platelet count \( (r = -0.815, p < 0.001, n = 10) \).

Fig. 3. Effect of transfer factor derived from the lymphocytes of control subjects, as well as from an ATP patient in remission, on leukocyte migration of leukocytes obtained from the buffy coat of ATP patients. Platelet membrane concentrations of 500 and 250 μg/ml protein were employed. "0" refers to migration index in the absence of transfer factor, "TF" to migration index in the presence of transfer factor.
MI from 0.84 ± 0.03 to 1.00 ± 0.09 at low concentration of antigen (p = 0.06). Similar results were also obtained from a 23-yr-old white female in remission for 4 yr, whose DLE increased the MI of an ATP patient from 0.83 to 0.98 at high concentration of antigen. Significant differences were not obtained with DLE obtained from a healthy control subject (Fig. 3, panels 1 and 2).

DLE was also prepared from leukocytes derived from a patient with severe ATP and tested on the leukocytes of two other patients with ATP, employing 500 µg/ml platelet membrane in the LMI assay. This DLE had no apparent effect on the migration index of the patients’ cells (mean MI of 0.82 ± 0.05 with DLE and 0.78 ± 0.10 without DLE).

Effect of DLE From a Patient With ATP and Thrombocytopenia on the MI of Normal Leukocytes Reacting to Platelet Membranes

DLE was obtained from a 42-yr-old white female patient with chronic ATP of 24 yr duration and a platelet count of 10,000/cu mm (who had given birth to a thrombocytopenic child in the past and was refractory to splenectomy). The addition of DLE from this patient to normal leukocytes significantly decreased the MI of their leukocytes reacting to platelet membranes at concentrations of 500 and 250 µg/ml (Figure 4). Thus, in 12 experiments, DLE treatment of normal leukocytes decreased the mean MI from 1.06 ± 0.04 to 0.88 ± 0.04 at a platelet membrane concentration of 500 µg/ml (p < 0.03), while it decreased the mean MI from 1.06 ± 0.05 to 0.9 ± 0.04 at a platelet membrane concentration of 250 µg/ml (p < 0.01).

DISCUSSION

We have demonstrated that leukocytes from patients with ATP exhibit the phenomenon of migration inhibition when incubated with homologous intact platelets or their membranes, whereas normal leukocytes do not. Since platelets were obtained from normal subjects, platelet–antibody complexes could not have been responsible. This reaction is specific, in that other antigens, such as keyhole limpet hemocyanin or tetanus toxoid, have no effect on the MI of ATP patients’ leukocytes. Thus, patients with ATP demonstrate cell-mediated immunity against normal platelets. Since HLA and Rh antigen specificities are not stimulatory in the direct LMI assay (which limits antigenic exposure to 45 min) using unsensitized leukocytes,13 it is likely that ATP patients have developed cell-mediated immune responses to common platelet membrane antigens, as is the case with antiplatelet humoral antibody.9,14 This observation is clinically relevant, since cell-mediated immunity, expressed as MI, is inversely related to the patients’ platelet counts.

The present findings, coupled with recent observations of a defective autologous mixed lymphocyte reaction in these patients,15 suggests that impaired immunoregulation may predispose ATP lymphocytes toward heightened reactivity to homologous platelets, with the resultant production of antiplatelet antibodies. These considerations suggested the possibility that lymphocyte–platelet antigen recognition might be abrogated with a specific lymphocyte suppressor factor for platelet membrane antigen(s).

An antigen-specific suppressor factor has recently been described in leukocyte dialysates containing transfer factor.10 (An antigen-specific inducer factor has also been described that can induce specific reactivity in naive lymphocytes.) The suppressor factor can abrogate specific reactivity of immune leukocytes. DLE with enhanced suppressor activity is usually obtained from a donor with exquisite delayed-type hypersensitivity reactions to the antigens studied.10 We therefore postulated that suppressor activity could be derived from DLE of patients with ATP in remission and inducer activity from DLE of patients with severe thrombocytopenia. Such proved to be the case. Thus, DLE from three patients with chronic ATP in remission was capable of correcting (reregulating) the MI generated by the reaction of ATP leukocytes with homologous platelets or their membranes; whereas DLE from nonimmune individuals had no effect. As a corollary, DLE from a patient with long-standing severe chronic ATP was capable of educating normal lymphocytes to react with homologous platelets or their membranes to generate an LMI reaction.
These results with DLE should be interpreted with caution because of the small number of DLEs tested. However, at least some ATP patients have a transfer factor derived from their leukocytes that is capable of modulating the in vitro leukocyte migration inhibition response of thrombocytopenic ATP patients as well as normal subjects.

These in vitro observations raise the possibility that DLE suppressor activity may be beneficial in the treatment of ATP.

REFERENCES

Leukocyte migration inhibition of buffy coats from patients with autoimmune thrombocytopenic purpura when exposed to normal platelets: modulation by transfer factor

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