Leukocyte-Platelet Interactions in a Murine Model of Chediak-Higashi Syndrome

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Chediak-Higashi (CH) syndrome, a genetic disease affecting man and other animals, is partially characterized by defective platelets that lack serotonin and dense bodies and by impaired leukocyte function where chemotaxis, degranulation, and bacterial killing are decreased. The effects of normal platelets containing serotonin and of reagent serotonin on the subnormal microbicidal activity of CH leukocytes were evaluated. The peripheral blood leukocytes of the beige mouse, an animal model with CH syndrome, were used with Staphylococcus aureus as the bacterial challenge. Addition of as few as two normal platelets/leukocyte resulted in normal levels of microbicidal activity of CH leukocytes. A similar normalization of leukocyte function was seen when 1-100-µM serotonin was added to the incubation mixture. Based on this work and work of others, a plausible explanation for these observations is that normal platelets interact with CH leukocytes, releasing serotonin, which results in reversal of the CH leukocyte defect in bacterial killing.

CHEDIAK-HIGASHI (CH) SYNDROME, which affects man and several other animal species, is inherited as an autosomal recessive trait. Its clinical constellation includes oculocutaneous albinism, a bleeding tendency, and compromised host defense against bacterial infections. The pathogenesis of the bleeding diathesis that is part of the CH syndrome has been partially elucidated. It has been shown that platelet serotonin and platelet dense bodies, the storage organelles for serotonin, are markedly decreased in CH platelets, and injection of serotonin in physiologic doses temporarily corrects the bleeding tendency of beige mice, one animal model for CH syndrome.

The pathogenesis of the compromised host defense against bacterial infections has been thought to be related to the abnormally enlarged granules of lysosome-containing cells. Specifically, polymorphonuclear leukocytes (PMN) show impaired chemotactic and microbicidal activity with decreased fusion of the enlarged lysosomal granules with phagocytic vacuoles.

The present studies were undertaken to test whether or not the lack of platelet serotonin might be causally related to the defect in leukocyte function in this CH model, the beige mouse. Previous studies have shown that normal platelets enhance PMN chemotaxis and potentiate bacterial killing. Our present studies showed a reversal of the characteristic defect in the ability of CH-PMN to kill phagocytosed Staphylococcus aureus when normal platelets or serotonin are present.
MATERIALS AND METHODS

The mice were C57B1/6 (bg/bg) and C57B1/6 (+/+), obtained from Oak Ridge Laboratories, Oak Ridge, Tenn., bred from original Oak Ridge stock, and C57B1/6J (+/+) obtained from Jackson Laboratories, Bar Harbor, Me. Whole blood was obtained by puncture of the retroorbital plexus, allowing 0.5 ml to drop through a heparinized microhematocrit tube into a 3.5-mm-diameter Petri dish that contained 0.5 ml Kreb's Ringer phosphate (KRP) buffer pH 7.4. A platelet count and total and differential leukocyte counts were performed on each diluted whole blood sample. *S. aureus* strain D2C was grown overnight in trypticase soy broth as previously described and was used as the test particle for the evaluation of intraleukocytic bacterial killing.

The diluted whole blood suspensions contained in a volume of 1 ml 2.5-5 × 10⁶ WBC, of which approximately 20% were PMN and 1-2 × 10⁸ were platelets. This was preincubated at 37°C in sterile 25-ml Erlenmeyer flasks for 15 min prior to the addition of Staphylococci at a ratio of bacteria:WBC of 2:1. Samples were removed just after the addition of bacteria and 30, 60, and 120 min later. Bacterial viability was determined according to previously described clonal culture methods.

The effect of normal (+/+) platelets on whole blood bacterial killing was determined by removing most of the native platelets by centrifuging the diluted whole blood at 150 g for 10 min. The platelet-rich supernatants contained 2-5 × 10⁵ platelets/ml and no PMN. Aliquots of these platelet-rich suspensions were added to other WBC-RBC sediments such that +/+ platelets were added to platelet-poor bg/bg leukocytes and bg/bg platelets were added to platelet-poor +/+ leukocytes. This resulted in the transfer of 10-20 bg/bg or +/+ platelets per PMN. In several experiments as few as two platelets/PMN were transferred. The resulting reconstituted samples were gently mixed and placed in sterile 25-ml Erlenmeyer flasks.

Platelet serotonin was estimated according to the method of Ashcroft et al. After centrifuging the diluted whole blood at 150 g for 10 min to obtain the platelet-rich supernatant, cold saline was added to bring the volume to 3 ml and the suspension was centrifuged at 1000 g for 10 min. The platelet button was suspended in 1 ml distilled water and lysed by three cycles of freezing and thawing. No measurable serotonin could be extracted from bg/bg platelets. Since the amount of serotonin that could be extracted from 2.5 × 10⁸ +/+ platelets/ml was 1 µg (5.5 µM serotonin), the addition of 2-5 × 10⁷ +/+ platelets resulted in the addition of 0.5-1 µM platelet serotonin.

To directly determine the effect of serotonin on intraleukocytic bacterial killing, serotonin as serotonin creatinine sulfate in a concentration range of 0.1-100 µM was added to diluted whole blood suspensions at the beginning of the period of preincubation. The serotonin (Sigma Chemicals, St. Louis, Mo.) was dissolved in KRP buffer pH 7.4.

The effect of 2-5 × 10⁴ platelets/ml and of serotonin 0.1-100 µM on bacterial viability in the absence of leukocytes also was evaluated. Significance of data was determined by Student's t test.

RESULTS

Evaluation of the microbicidal activity of bg/bg and +/+ diluted whole blood samples confirmed that the microbicidal activity of bg/bg leukocytes was significantly impaired when compared to the activity of +/+ controls in six of six experiments. These results (Fig. 1) show that after 2 hr of incubation bg/bg leukocytes killed only 70% ± 1.5% of the bacterial inoculum while +/+ leukocytes killed 89% ± 0.7% (p < 0.01). Significant impairment of bacterial killing also was seen at the earlier sampling times. There was no killing by cell-free diluent (KRP) buffer with either bg/bg or +/+ mouse plasma.

In order to determine whether or not normal platelets influence bg/bg leukocyte function, the separated +/+ platelets were added to bg/bg PMN. The effect of this cross-exchange of 10-20 platelets/PMN is shown in Fig. 2. The addition of +/+ platelets to bg/bg blood resulted in enhanced bacterial killing by bg/bg leukocytes to the level of that of normal PMN with normal platelets, i.e., 88% ± 0.5% of the bacterial inoculum was killed when bg/bg leukocytes were incubated with +/+ platelets while only 70% + 1.5% was
Fig. 1. Microbicidal capacity of peripheral blood leukocytes in diluted whole blood incubated with S. aureus shown as decrease in number of viable staphylococci as function of time. Normal leukocytes, .; CH leukocytes, . Recorded values, means ± SEM of six experiments. Effect of cell-free plasma buffer also shown: normal plasma, ; CH plasma, .

Fig. 2. Effect of adding 10–20 normal platelets per CH leukocyte (△) and 10–12 CH platelets per normal leukocyte (■) on microbicidal capacity of these leukocytes in diluted whole blood, along with their microbicidal activity in presence of isologous platelets. CH leukocytes with CH platelets, ; normal leukocytes with normal platelets, ; microbicidal effect of platelets alone, .. Mean ± SEM.
killed when only isologous platelets were present ($p < 0.001$). Preliminary data suggest that correction of the defective bacterial killing of $bg/bg$ leukocytes also occurred when as few as two $+/+$ platelets (0.1–0.2 $\mu M$ serotonin equivalent) per $bg/bg$ PMN were used. These experiments showed a range of correction of 80%–100% of control levels. There was no loss of function of normal PMN when $bg/bg$ platelets were added.

Cell-free plasma obtained by removal of platelets by centrifugation, with cross-substitution of $bg/bg$ and $+/+$ platelet-free plasma, failed to significantly influence bacterial killing by either $bg/bg$ or $+/+$ leukocytes (Fig. 3). Other controls measured whether or not platelets in the absence of other leukocytes were able to effect bacterial killing. These controls showed that platelets in the absence of other leukocytes did not kill significant numbers of staphylococci (Fig. 2). These experiments showed a 16% ± 7.6% decrease in bacterial viability after 2 hr of incubation ($p > 0.05$).

The effect of adding serotonin to diluted $bg/bg$ blood is shown in Fig. 4A. Bacterial killing by $bg/bg$ leukocytes was increased from 70% ± 1.5% to 89%–91% with serotonin concentrations of 1, 10 (not shown in Fig. 4A), and 100 $\mu M$ ($p < 0.01$). Thus complete correction of the $bg/bg$ microbicidal defect was associated with concentrations of serotonin between 1 and 100 $\mu M$. At concentrations of 0.6 or 0.1 $\mu M$ no significant correction was observed. At 120 min serotonin had no significant effect on the already high microbicidal activity of $+/+$ cells, but increases in early killing were observed (Fig. 4B). Serotonin in the absence of leukocytes failed to kill bacteria (Fig. 4A).
DISCUSSION

Bacterial killing by Chediak-Higashi leukocytes was increased to normal values by normal platelet-rich plasma or by serotonin. This improvement of bg/bg leukocyte function in the presence of normal platelets suggests that a platelet leukocyte interaction mediates this effect, since neither plasma nor platelets per se in the absence of leukocytes effected bacterial killing. Also, the transfer of cell-free plasma from +/+ mice to bg/bg leukocytes failed to correct the bg/bg functional deficit. The absence of serotonin in CH platelets and the observation that the abnormal bacterial killing by CH-PMN was corrected by small amounts of serotonin lend support to the idea that the platelet component involved in the interaction may be serotonin. These findings suggest that the platelet serotonin deficiency and the leukocyte dysfunction could be related, since at least one of the leukocytic abnormalities (defective bacterial killing) is corrected when the leukocytes are incubated in the presence of normal platelets or of serotonin. However, while these data have clearly shown that normal platelets or serotonin corrects the abnormal bacterial killing of CH leukocytes, firm documentation that the platelet effector is serotonin remains largely circumstantial. The fact that +/+ leukocyte bacterial killing is not impaired following the transfer of bg/bg platelets could be due to the previously documented incomplete removal of native platelets, where the remaining +/+ platelets are sufficient for normal function.

Our data suggest that very few platelets are necessary for effective platelet leukocyte interaction. The correction of the bacterial killing defect by serotonin
does not appear to be linearly dose related but rather shows a threshold effect. This relationship was seen also in the serotonin correction of the bg/bg bleeding time abnormality. Our data seem to show that smaller amounts of platelet serotonin are necessary to correct the functional defect than of serotonin alone. This can be explained by the possibility of higher local concentrations of serotonin in cell-cell interactions. Also, it is possible that the serotonin extracted from platelets does not reflect all of their available serotonin. Northover in 1961 provided some support for the concept that serotonin affects leukocyte function by showing that serotonin at physiologic concentrations would enhance phagocytosis by PMN.

Rationale for our hypothesis can be constructed from a logical combination of several observations in the literature. Abnormalities of the cellular structural elements of PMN have been shown to be associated with impaired host defense against bacterial infection. The functional integrity of one of these subcellular elements, cytoplasmic microtubules, is known to be dependent on cGMP. Several recent studies have shown that cGMP and substances that raise cellular cGMP or lower cellular cAMP correct many of the CH defects, including chemotaxis, degranulation, bacterial killing, and the defects in microtubule polymerization. One of the substances that enhances cGMP formation in normal cells is serotonin. The evidence that cGMP and cytoplasmic microtubules play an important role in chemotactic activity and in granule movement within cells, together with the finding that both of these PMN functions and microtubules are abnormal in CH leukocytes, suggests a causal link between the observed platelet and leukocyte abnormalities that stems from lack of platelet serotonin. Thus a reasonable interpretation of our data would be that the addition of normal platelets or serotonin to bg/bg leukocytes enhances the formation of cellular cGMP, which promotes the polymerization of tubulin to microtubules, resulting in reversal of the abnormal microbicidal activity. Since previous studies have shown that nearly all of the circulatory serotonin is located in blood platelets, it is probable that the platelet defect plays an important role in the pathophysiology of the impaired host defense characteristic of human CH disease. It is also reasonable to extrapolate these data, together with the evidence that serotonin raises cGMP in normal leukocytes, to suggest that an interaction of normal platelets and PMN leukocytes may play a role in normal host defense. While normal leukocytes are thought to function maximally in the absence of platelets, they never actually function this way. Even in severe thrombocytopenia, two platelets per leukocyte usually are present. Also, leukocytes for studies in vitro generally are taken from subjects with normal platelet counts; these leukocytes may be primed to function optimally.

A role for platelets as a physiologic effector of leukocyte function is far from clear. However, the slowly accumulating evidence, both from this paper and from the literature, suggests that a closer look at such an interrelationship is necessary.

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