Decreased Nucleotide and Serotonin Storage Associated With Defective Function in Chediak–Higashi Syndrome Cattle and Human Platelets

By Thomas G. Bell, Kenneth M. Meyers, David J. Prieur, Anthony S. Fauci, Sheldon M. Wolff, and George A. Padgett

Prolonged mean template bleeding time of 14 min observed in seven cattle with the Chediak–Higashi syndrome (CHS) prompted the examination of platelet function in these animals. There was no distinguishable difference in concentration of circulating platelets between CHS and control cattle. CHS bovine platelets failed to aggregate in vitro in response to concentrations of acid-soluble collagen which induced aggregation in all normal samples. The primary platelet response to 5 μM ADP was normal in CHS cattle. A markedly decreased amount of serotonin (1.2% of normal) was detected in CHS bovine platelets. Bovine CHS platelet ATP and ADP contents were significantly less than normal, and the ATP/ADP ratios were 5.04 in normal and 29.38 in CHS platelets. Results of these animal investigations prompted a similar study of two patients with CHS. In humans, an increased bleeding time greater than 15 min and an in vitro impaired aggregation response to acid-soluble collagen and 5 μM adrenaline were discovered. Both ATP and ADP were reduced in CHS human platelets, and the ATP/ADP ratio was 3.96, compared to a ratio of 1.52 for platelets of two normal subjects. These findings suggested the presence of a "storage pool disease" of CHS platelets.

The Chediak–Higashi Syndrome (CHS) is a genetic disorder in man,1 mink,2 cattle,3 mice,4 cats,5 and killer whales,6 with an autosomal recessive mode of inheritance. With CHS there are oculocutaneous albinism, recurrent pyogenic infections, enlarged granules in most granule-containing cells, and in at least man, mice, cats, and cattle, a hemorrhagic tendency.7 Even though there is a clinically apparent bleeding tendency in CHS mink and cattle, a detailed study of the coagulation and fibrinolytic systems in these animals has failed to disclose a cause for the hemorrhagic tendency.8 These animals have normal platelet counts. Whole blood serotonin of CHS humans,9 cattle, and mink10 has been shown to be markedly reduced. Also, it has recently been reported that there is a decreased total serotonin uptake by platelet-rich plasma (PRP) of CHS cattle.11 The initial rate of serotonin uptake, however, is normal.

The reports and observations of a hemorrhagic tendency,7 in association with normal soluble clotting factor parameters and normal platelet counts coupled with low levels of blood serotonin and reduced PRP serotonin uptake, sug-
uggested the presence of a platelet defect in CHS. To characterize the suspected platelet abnormality, several biochemical and functional parameters of CHS and normal bovine platelets were defined. The results of the bovine studies prompted a similar study of platelets from two human subjects with CHS.

About half of the CHS patients enter a lymphoma-like "accelerated phase" accompanied by thrombocytopenia. The hepatosplenomegaly, lymphadenopathy, and pancytopenia seen in some CHS patients with the accelerated phase have not been present in the CHS subjects studied. This report compares the CHS bovine and human platelet defect and attempts to relate the clinically observed hemorrhagic diathesis in this syndrome to the defective function of the CHS platelets.

**MATERIALS AND METHODS**

**Cattle**

The CHS cattle were from the experimental CHS Hereford herd maintained by Washington State University. The normal cattle were from registered Hereford stock. All of the cattle were age-matched so that each CHS cow was paired with a normal cow whose age was within 2 mo. Ages ranged from 4 mo to 5 yr.

**Human Subjects**

Healthy laboratory personnel served as normal controls. Two brothers with CHS, L.E. and L.A., who have been previously described in detail, were studied when temperatures were normal and no known infection was present. At the time of study, L.E. was a 25-yr-old male who had been diagnosed as having CHS at the age of 15 yr on the basis of partial albinism and characteristic leukocyte granules. L.E. has never manifested signs of the accelerated phase, although severe pyoderma existed for a period of over a year between ages 16 and 17. Except for epistaxis at an early age, the history was negative for a hemorrhagic diathesis. Seven days preceding, and during the current study, drugs affecting platelet function were withheld.

L.A. is a 24-yr-old male, the brother of L.E. Severe pyoderma at the age of 11 led to the diagnosis of CHS. L.A. also has not manifested the accelerated phase. Pneumonia, severe pyoderma, a nuchal carbuncle, and periodontitis are characteristic of the history, although the out-patient period of 2 mo preceding the present study was uneventful. Occasional epistaxes between the age of 3 and 15 yr requiring professional care were reported by the parents. During the 7 days preceding, and during the current investigation, drugs affecting platelet function were withheld.

**Blood and Platelet Collection, Storage, and Enumeration**

All collections were from peripheral veins, the jugular vein in cattle and the median cubital in humans. A two-syringe technique was used; the blood in the first syringe was discarded. Collection equipment was plastic or siliconized glass. Anticoagulation was achieved with 3.8% trisodium citrate solution, pH 7.4. One volume of the citrate solution was mixed with 9 volumes of human blood and 18 volumes of bovine blood. PRP was obtained by centrifuging cow blood for 45 min and human blood for 12 min at 180 g. Platelet-poor plasma (PPP) was obtained by the centrifugation of human and bovine PRP at 5000 g for 20 min and 30 min, respectively. Samples used for aggregation studies were stored in 12-ml polycarbonate tubes in 10-ml aliquots at 25°C and used within 4 hr. Samples for serotonin or adenine nucleotide assay were processed within 15 min of collection and stored at -20°C in 3-ml plastic tubes (see serotonin and nucleotide assay below). Platelet counts of whole blood from the same sample were performed in RBC chambers with phase microscopy, followed by PRP platelet counts and sizing obtained electronically while platelets were suspended in Isoton (Coulter Electronics, Hialeah, Fla., and Particle Data, Elmhurst, Ind.).
Aggregation Function

Trisodium citrate PRP was utilized for study of aggregation function, with platelet counts ranging from 323 to 595.5 x 10^9/liter in human beings and 488 to 1332 x 10^9/liter in cattle. Aggregometry on human samples was conducted in a Chrono-log aggregometer (Chrono-log, Broomall, Pa.) and cattle aggregometry in a spectrophotometer (Bausch & Lomb, Ithaca, N.Y.) adapted for turbidometric function. The techniques and procedures of Holmsen et al. were followed. In each case, the PRP response to 5 μM ADP (final concentration) was followed in other samples by ADP addition of one-half the previous concentration until incomplete aggregations occurred, as determined by a recorded return toward preaddition light transmittance. Each subject's PRP was also tested with 0.05% collagen (final concentration) prepared as described by Holmsen et al. Finally, PRP was tested with 5 μM adrenaline. Maximal extent of aggregation was expressed as the total decrease in percentage light transmission (660 nm) in arbitrary chart units after addition of the inducing agent. Initial rates of aggregation were measured in arbitrary chart units by fitting a line from the point of addition to the aggregation tracing extending through 1 min.

Bleeding Time Determination

Bleeding time determination, conducted according to established methods, requires that the appendage incised be cuffed so that stasis of venous flow occurs while arterial supply continues. In humans, the cuff pressure on an arm was maintained at 40 mm of mercury. In cattle, the shaven base of the tail was utilized as the site for bleeding time determination. A cuff was applied so that venous engorgement occurred while the pulse distal to the cuff was palpable. After incision, the time until cessation of bleeding was measured.

Assay of Serotonin

Platelet serotonin was quantitated using the fluorometric assay of Yuwiler et al., a modification of Bogdanski's method. A PRP sample of at least 5 ml and of known volume was sedimented according to the method of Holmsen after the addition of 1/20 volume of 0.1 M K₂EDTA. The supernatant solution was decanted and discarded, and the sample was stored at -20°C until assay. Preliminary experiments detected 3% or less of total serotonin fluorescence in the supernatant. The platelet sediment was restored to the prestorage volume by the addition of cold (4°C) 0.1 M K₂EDTA in 0.85% NaCl, thus eliminating dilution factors. This suspension was used in the serotonin assays. Serotonin was expressed as nmoles/10^11 platelets.

Nucleotide Assay

The determination of nucleotide content of ethanol-extracted platelets from counted PRP was performed precisely as described by Holmsen et al., with the exception that the firefly lantern (Sigma Chemical Co., St. Louis, Mo.) was subjected to mild homogenization for 15-30 sec in a glass Tenbrook homogenizer. This treatment resulted in a very slight reduction of activity but greater consistency in a series of determinations with a given suspension of firefly lantern extract. PRP samples were stored at 0°-4°C after mixing for at least 5 min and not longer than 10 min after the addition of K₂EDTA-ethanol solution before centrifugation. The use of K₂EDTA (Cambridge Chemical Products, Fort Lauderdale, Fla.) in the preparation of EDTA-ethanol in place of Na₂EDTA allowed storage at -20°C of this reagent. Preliminary trials comparing K₂EDTA and Na₂EDTA disclosed no effect on ATP or ADP measurement. The amount of the ethanolic extract assayed was increased from 20 to 100 μl in those samples with levels of ADP less than 1.0 μmole/10^11 platelets.

Statistical Analysis

Results were analyzed by means of Student's t test (unpaired) and, unless otherwise stated, expressed as means ± SD. Differences with p < 0.01 were considered to be significant.
RESULTS

The results of triplicate bleeding times of cattle and human beings are illustrated in Fig. 1. Seven CHS cattle had a mean bleeding time of 14.5 ± 2.7 min, while the bleeding times of seven control cattle were 3.8 ± 1.6 min (p < 0.01). The platelet counts were not significantly different (CHS 583 ± 85 compared to

Table 1. Aggregation Response of PRP From CHS and Control Bovine and Human Subjects

<table>
<thead>
<tr>
<th></th>
<th>5.0 µM ADP</th>
<th>0.05% Collagen*</th>
<th>5.0 µM Adrenaline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max. Extent</td>
<td>Initial Rate</td>
<td>Max. Extent</td>
</tr>
<tr>
<td>Bovine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>5.3</td>
<td>4.6</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>3.9</td>
<td>7.8</td>
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<tr>
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<td>8.9</td>
<td>5.4</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>8.4</td>
<td>5.7</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>5.7</td>
<td>5.1</td>
<td>8.1</td>
</tr>
<tr>
<td>Mean ± (SD)</td>
<td>6.7 ± 1.8</td>
<td>5.0 ± 0.7</td>
<td>8.2 ± 0.6</td>
</tr>
<tr>
<td>CHS</td>
<td>7.5</td>
<td>5.5</td>
<td>1.5</td>
</tr>
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<td></td>
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<td>5.0</td>
<td>1.0</td>
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<td></td>
<td>5.8</td>
<td>4.8</td>
<td>5.5</td>
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<td></td>
<td>6.4</td>
<td>5.6</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>5.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Mean ± (SD)</td>
<td>6.3 ± 0.7</td>
<td>5.2 ± 0.3</td>
<td>2.2 ± 1.9</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
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<tr>
<td></td>
<td>6.9</td>
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<tr>
<td>Mean</td>
<td>7.3</td>
<td>6.1</td>
<td>7.4</td>
</tr>
<tr>
<td>CHS</td>
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<td>3.6</td>
<td>0.0</td>
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<td></td>
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<td>4.7</td>
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<tr>
<td>Mean</td>
<td>6.3</td>
<td>3.9</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Each paired column presents maximal extents and initial (first minute) rate aggregation in response to the indicated final concentration of aggregating agent. Maximum extents and initial rate of aggregation are given in arbitrary chart units and chart units per minute, respectively. Dashes indicate no aggregation response.

*Each bovine normal sample responded with full aggregation to 0.01% collagen while no bovine CHS sample responded.
NUCLEOTIDE AND SEROTONIN STORAGE IN CHS

Fig. 2. Aggregation patterns of PRP from CHS and normal individuals in response to 5 μM ADP, 0.05% collagen, and 5 μM adrenaline. The tracings represent the change in the 660 nm light transmission as a function of time (10% change in transmission indicated by "Δ Trans" and the time in minutes by "min."). The PRP system contained 0.45 ml (human) or 1.9 ml (bovine) PRP, to which was added 0.05 or 0.1 ml aggregating agent. Bovine PRP contained 0.19% and human 0.38% trisodium citrate. Bovine PRP is not responsive to adrenaline (see text).

with normal, 518 ± 169 x 10⁹/liter of blood). Normal human subjects tested had a triplicate mean bleeding time of 6.0 and 5.0 min. Two CHS patients had a triplicate mean bleeding time of 15.8 and 23.4 min. Whole blood platelet counts of the four humans (CHS and control) were 428 and 533 (normal), and 420 and 574 (CHS) x 10⁹/liter.

Table 1 lists data from in vitro aggregation experiments, and typical individual aggregation responses are reproduced in Fig. 2. Maximal response to ADP was decreased slightly in the CHS individuals of both species. The initial rate of ADP induced aggregation was reduced in CHS human PRP, but was not considered significantly different from the control rate. Biphasic aggregation was observed only in control human PRP exposed to ADP levels between 0.75 and 1.5 μM and to 5 μM adrenaline.

The extent of aggregation and its rate following 0.05% collagen addition were reduced markedly in CHS PRP obtained from both humans and cattle. Furthermore, normal PRP began to aggregate within 30–80 sec after collagen addition, while CHS samples responded only slightly, if at all, within the first 60 sec. Normal bovine PRP responded to 0.01% collagen as readily as to 0.05%. CHS bovine PRP evidenced no response to 0.01% collagen.

In control human PRP, 5.0 μM adrenaline regularly produced a biphasic aggregation response with the first phase lasting 60–90 sec and with complete aggregation occurring within 5 min. CHS human PRP did not respond to 5.0 μM adrenaline, whereas one of the two CHS patient's PRP responded at twice the concentration of adrenaline.23 Neither control nor CHS bovine PRP aggregated in response to the addition of adrenaline from 5 to 500 μM.24

In Table 2, the analyses of serotonin, ATP, and ADP from PRP of an equal number of CHS and control individuals, ten bovine and four human, are presented. The nucleotide data are charted in Fig. 3. There are marked reductions of serotonin and ADP in both human and bovine CHS PRP. Platelet serotonin concentrations in CHS cattle were only 1.2% of controls, whereas human CHS samples contained 25.6% of human controls.
Table 2. Platelet Serotonin, ATP, and ADP From CHS and Control Bovine and Human Subjects

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>CHS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ser. (nmoles/10^11 cells)</td>
<td>ATP (µmoles/10^11 cells)</td>
</tr>
<tr>
<td>Bovine</td>
<td>787.45</td>
<td>3.86</td>
</tr>
<tr>
<td></td>
<td>390.05</td>
<td>3.42</td>
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<tr>
<td></td>
<td>900.77</td>
<td>3.56</td>
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<tr>
<td></td>
<td>449.50</td>
<td>3.60</td>
</tr>
<tr>
<td></td>
<td>512.29</td>
<td>3.04</td>
</tr>
<tr>
<td>Mean</td>
<td>608.01 (± SD) (223.44)</td>
<td>3.50</td>
</tr>
<tr>
<td>Human</td>
<td>148.83</td>
<td>5.26</td>
</tr>
<tr>
<td></td>
<td>126.97</td>
<td>4.93</td>
</tr>
<tr>
<td>Mean</td>
<td>137.90</td>
<td>5.10</td>
</tr>
</tbody>
</table>

The experimental procedure is described in the text. Each tabular entry represents the mean of triplicate assays from the individual.

The adenine nucleotides, ATP and ADP, were reduced in both groups of CHS PRP analyzed. ATP in CHS human samples was reduced to 47.3% of controls, whereas bovine CHS ATP was reduced to 54.9%. On the other hand, ADP in human CHS platelets was reduced to 22.5%, whereas bovine CHS ADP was decreased to 13.3% of controls. The ATP/ADP ratios in normal human and bovine PRP were 1.52 and 5.0, respectively. CHS samples had elevated ratios of 3.96 in human and 29.38 in cattle PRP.

Fig. 3. Mean ATP and ADP from normal and CHS human and bovine platelet-rich plasma. The numbers in parentheses represent the number of patients or animals within each group, and the vertical lines represent the standard deviations of the means in the bovine and the ranges in the human.
DISCUSSION

During the past 7 yr, 20 of 56 deaths in the herd of CHS cattle maintained at Washington State University could be directly associated with hemorrhage.7 In 27 deaths among CHS children, hemorrhage was considered to be the cause of death in 4 and a significant clinical factor in 13 patients. Of 42 clinical reports of CHS children, 24 assessed hemorrhage and, of those, 13 reports indicated that a hemorrhagic tendency was present.7 The pancytopenia accompanying the accelerated phase accounts for some reports in humans;14 however, the finding of prolonged bleeding time and no thrombocytopenia in our and other CHS patients,25 points to a clinically significant hemorrhagic diathesis.

The bleeding tendency in the CHS cattle is often manifested clinically by fibrosis in resolution of large subcutaneous accumulations of blood and by the production of numerous, firm subcutaneous nodules at sites of previous trauma. Penetration of the skin at the time of injury has often resulted in secondary infection during the resolution phase. These complications have delayed recognition of the hemorrhagic tendency.

Bleeding time is related to platelet function.18 In this study, prolonged CHS bleeding times have been investigated by characterization of platelet function in vitro. The agents used to assess platelet function allow inferences as to the nature of platelet defectiveness.26 Collagen releases stored (storage pool) ADP during the platelet release reaction in aggregation. ADP and adrenaline have direct effects on platelets which initiate aggregation. These two substances, in appropriate concentrations, may induce a biphasic aggregation response due to the initial effect of the introduced substance, followed by a secondary release of platelet storage pool ADP. A decreased or absent pool of secretable (storage) adenine nucleotides thus manifests functionally as a reduction in response to collagen and a loss of biphasic response to ADP and adrenaline in vitro. These characteristic responses are similar whether there is a deficit in or a failure of release from (as in aspirin-induced defect) the storage pool.

The extent of the decrease in platelet serotonin and nucleotides in bovine CHS platelets is, compared to storage pool diseases described in man,26 relatively massive. Previous investigation of CHS bovine platelets has revealed normal initial platelet uptake of serotonin but failure of storage.11 Recent studies from our laboratory have shown that CHS human platelets appear to have normal transmembrane serotonin transport. Although these platelets appear to have fewer dense granules, they will release the dense granule serotonin that is present in response to thrombin.27

The nucleotide analyses of CHS platelets strongly indicate marked reduction in the storage pool based upon the increased ratio of ATP/ADP. The findings have functional significance. Human platelets are known to have about two-thirds of the total adenine nucleotides in the storage pool. Of this amount, about 80% is ADP, which accounts for over three-fourths of platelet ADP.28,29 A reduction of ADP measured in CHS platelets suggests that there is less than normal ADP available for release upon stimulation.25

While less is known of the bovine platelet, most nonprimate species have normally higher ratios of ATP/ADP than man.24 Except for cats, nonprimate platelets do not respond to ADP or adrenaline with biphasic aggregation in vitro. The in vivo significance of these differences is not clear.
The inability of CHS bovine platelets to store serotonin does not correspond quantitatively to the nucleotide deficit. It has been proposed that a micellar complexing of adenine nucleotide, cations, and serotonin occurs in storage organelles of platelets. Furthermore, it has been recently determined that there is an association between Ca\(^{2+}\)-ADP and serotonin-ATP in storage. Non-labeled ATP assay of platelets (as in this study) measures metabolic as well as storage pool ATP, and, consequently, only when bovine platelet metabolic and storage pools are experimentally defined could one expect to detect decreased storage ATP matching the magnitude of the serotonin deficit.

While the storage mechanism for the granular nucleotides is unclear, there is evidence that newly released platelets may already contain storage pool nucleotides, and that exchange with the metabolic pool is slow. Several possibilities explaining the observed defect in CHS exist: (1) megakaryocytes are unable to produce platelets with dense granule (storage) organelles; (2) platelets are released with fewer (but normal) dense granules; or (3) the platelets are released with normal numbers of dense granules, but the storage mechanism(s) is abnormal. Initial electron micrographic studies on CHS animal platelets do not clarify the situation. While there appear to be fewer dense bodies, one can only be certain of the identification of those organelles containing electron dense material.

The use of animal model systems to study disease processes is a well-established practice. However, application of data across species lines is a problem, and it is important that several species with a particular defect be studied or idiosyncratic differences within a species may mask the effect under investigation. In CHS, one is provided with five storage pool disorders in diverse species that have markedly different normal platelet nucleotide levels and ATP/ADP ratios. These CHS animals have been well defined genetically, clinically, and morphologically, and in terms of functional cellular defects. The application of new methodology to CHS platelets should allow significant information concerning platelet function to be translated into generally applicable principles. Thus, despite species differences, animal models of human platelet storage diseases such as the Fawn-Hooded rat and the CHS are likely to contribute to an understanding of platelet storage and release mechanisms.

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