In Vitro Platelet Abnormality in Adenosine Deaminase Deficiency and Severe Combined Immunodeficiency

By Choon H. Lee, Susan P. Evans, Maurice C. Rozenberg, Aldo S. Bagnara, John B. Ziegler, and Martin B. Van der Weyden.

The platelets of an infant with severe combined immune deficiency and adenosine deaminase deficiency showed markedly diminished responses to ADP-induced aggregation in vitro. This abnormality was corrected by the addition of purified adenosine deaminase in vitro. Exogenous adenosine added to platelet-rich plasma caused markedly prolonged inhibition of ADP-induced aggregation. This was shown by isotopic studies to be due to slow clearance of adenosine and hence persistence of this nucleoside. Direct assay for adenosine deaminase in plasma and platelet lysates of the patient confirmed the very low activity of this enzyme. Raised cAMP levels were demonstrated in his platelets. The deranged adenosine metabolism and raised cAMP in the platelets of this child with severe combined immunodeficiency may explain the altered response to ADP. Despite the in vitro platelet aggregation abnormality, the patient had no clinical evidence of impaired hemostasis.

The syndrome of severe combined immune deficiency (SCID) with adenosine deaminase (ADA) deficiency was first described in 1972. A 4-mo-old infant with this syndrome was referred to us for diagnosis and management. ADA activity was found to be undetectable in his erythrocytes and serum. He was commenced on a course of red cell transfusion therapy similar to that of Polmar et al., providing us with sufficient blood to study his platelet function and metabolism. A full report of his clinical course is to be published elsewhere.

ADA converts the purine nucleoside adenosine to inosine. In ADA-negative SCID, the enzyme deficiency has been demonstrated in a wide variety of tissues, including sera, erythrocytes, leukocytes, spleen, liver, and fibroblasts. It is reasonable to expect that the platelets will also lack the enzyme, although this fact has not been specifically recorded. Lack of this enzyme would block a major pathway of adenosine metabolism in platelets and other tissues and could conceivably lead to accumulation of adenosine in vivo. Adenosine is known to inhibit ADP-induced platelet aggregation in vitro and in vivo. This article reports the patient’s platelet responses to various aggregating agents in vitro and the ADA activity of his platelets and plasma. We also studied the inhibitory effect on ADP-induced aggregation by exogenous adenosine, and this was correlated with simultaneous...
metabolic study of PRP using [8-¹⁴C]-adenosine. The correction of an aggregation abnormality of the patient's platelets to ADP-induced aggregation by highly purified ADA was then demonstrated. Adenine nucleotide levels in his platelets were measured by high-pressure liquid chromatography and cAMP levels by radioimmunoassay, as these parameters could have significant implications for the inhibitory role of adenosine. During the preparation of this manuscript, the first report of a platelet aggregation defect in ADA-negative SCID was published.⁷

**MATERIALS AND METHODS**

All the studies on the patient reported in this article were carried out just prior to each exchange transfusion; in all instances circulating erythrocyte ADA activity was less than 15% of the mean value for normal subjects. Blood was collected by clean venipuncture into plastic tubes containing 0.10 volume of 3.8% sodium citrate. Platelet-rich and platelet-poor plasmas were then obtained by centrifugation at 400 g and 1000 g, respectively, for 10 min at 20°C. The platelet-rich plasma (PRP) was then adjusted to a count of 200-250 X 10⁹/l for aggregometric studies. All studies were completed within 2½ hr of blood collection. Aggregating agent (0.1 ml) was added to PRP (0.9 ml) in the cuvette of an Eel titrator kept at 37°C, and the change in optical density was recorded on a Servoscribe RE 511.20. Both the rate of the aggregation response %Δ O.D./min and the maximum aggregation achieved %Δ O.D. were calculated. Aggregating agents tested (final concentrations given) included collagen (1 mg/ml), ristocetin (1.5 mg/ml), bovine fibrinogen (1.2 mg/ml), adrenalin (50 µg/ml), and ADP (1.25-µM, 2.50-µM, and 5.00-µM) (2.5 µg/ml = 5-µM).

Inhibitory effects of exogenous adenosine were studied by incubating PRP with adenosine (12.5-µM) at 37°C in stoppered plastic tubes. At timed intervals, continuing up to 90 min, ADP-induced aggregation was measured (ADP 5.0-µM) as above. Inhibition was measured by comparing the aggregation response with that obtained with PRP incubated with the same small volume of Owren's buffer for a similar time period (Fig. 1A). At the same time the metabolism of adenosine in PRP was studied using [8-¹⁴C]-adenosine, also at 12.5-µM, under the same conditions. Samples were deproteinized at timed intervals with 0.5-M perchloric acid at 0°C. This was subsequently neutralized with 4.5-M KOH/1.0-M KHCO₃, and 5 or 10 IIl of supernatant were used for chromatographic analysis. Resolution of purine compounds was obtained either by two-dimensional paper chromatography⁴ or by thin-layer chromatography on PEI-cellulose (Machery-Nagel).⁸ Spots were visualized under UV light, cut out and placed in 4 ml of scintillation fluid (p-terphenyl in toluene or PCS Amersham), and counted with about 55% efficiency in a Packard Tricarb beta scintillation counter.

The effect of exogenous purified ADA (Sigma type III) on ADP-induced platelet aggregation was also studied. PRP was incubated at 37°C with ADA (at a final activity of 2 IU/ml) for 10 min, and ADP-induced aggregation was compared with that of PRP incubated with Owren's buffer for a similar time. All three concentrations of ADP were employed.

Platelet lysates were prepared from PRP (anticoagulated with 0.075 volume of 0.077-M sodium EDTA, pH 7.4) by washing a known number of platelets (counted by a Coulter thrombocounter) twice with a mixture of 0.154-M NaCl, 0.154-M TRIS (pH 7.4), and 0.077-M sodium EDTA (90:8:2 by volume). The platelet button was freeze-thawed three times in dry ice/acetone, and a small volume of TRIS saline (20-M TRIS, pH 7.4) was added to reconstitute a crude platelet lysate. ADA activity in platelet lysates was measured in a standard assay. The ADA assay mixture, kept at 37°C, consisted of 50 µl of platelet lysate (approximately 1 X 10⁹ platelets) and 25 µl of 1-mM [8-¹⁴C]-adenosine (10 mCi/m mole). The reaction was stopped after 15 min by the addition of 25 µl of 3-M HClO₄ at 0°C. The contents were then neutralized with 15 µl of 4.5-M KOH/1.0-M KHCO₃, and chromatographic separation of inosine and hypoxanthine from adenosine was achieved with one-dimensional paper chromatography⁴ using only the first solvent or with thin-layer chromatography on PEI-cellulose (Machery-Nagel).⁸ Spots were visualized under UV light, cut out and placed in 4 ml of scintillation fluid (p-terphenyl in toluene or PCS Amersham), and counted with about 55% efficiency in a Packard Tricarb beta scintillation counter.

Plasma ADA activity was determined as described above for platelet lysates, but using 50 µl of citrated plasma. In calculating the plasma ADA activity, the diluting effect of citrate was taken into consideration, and the results were expressed as micromoles per hour per 10⁹ platelets.

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Adenine nucleotide levels in platelets were measured in perchloric acid extracts of platelets by high-pressure liquid chromatography using an Altex model 110 system fitted with a Partisil-10 SAX anion-exchange column. Elution was performed at a flow rate of 2 ml/min using a 20-min linear gradient from 10-mM to 500-mM NH₄H₂O₄ (pH 4.0). Adenine nucleotide peaks were detected at 254 nm and were quantified using an Autolab Integrator with peak area calibration curves constructed from standard nucleotide solutions. Cyclic AMP levels in platelets were measured in similar platelet extracts using the cAMP radioimmunoassay kit (Amersham).

RESULTS

Table I shows the aggregation responses. There were reduced rates and extents of aggregation with all concentrations of ADP used and essentially normal aggregation behavior with the other agents. Disaggregation occurred even at high levels of ADP (5.0-μM).

Figure 1B shows the rates of recovery from adenosine inhibition in 6 normal subjects, the patient’s mother (heterozygote), and the patient. ADP-induced platelet aggregation (ADP = 5.0-μM) returned to normal at between 40 and 90 min with platelets from normal subjects and from the mother. There was virtually no recovery from adenosine inhibition with the patient’s platelets studied on two different occasions.
Figure 2 shows the results of $^{14}$C tracer studies on the PRP of the patient and of a normal subject. $[8-{^14}C]$-adenosine was rapidly metabolized with reciprocal accumulation of $[8-{^14}C]$-inosine and hypoxanthine and some incorporation into nucleotides, as expected in normal PRP. Deamination of adenosine was slow in the patient's PRP, with appreciable incorporation of adenosine into adenine nucleotides. These results indicate that the patient's PRP has exceedingly low ADA activity. This finding is substantiated by direct measurements of ADA activity (Table 2), which showed that there was indeed greatly decreased ADA activity in the patient's plasma and platelet lysates.

ADP-induced aggregation was corrected to within the lower limit of the normal response on addition of exogenous ADA (2 IU/ml) (Fig. 3, Table 3). The corrective effect of ADA on platelet aggregation was abolished when the enzyme was...
Table 2. ADA Activity in Platelet Lysates and Plasma of the Patient With SCID, His Parents, and Normal Controls

<table>
<thead>
<tr>
<th></th>
<th>Platelet lysates (μmoles/hr/10^11 platelets)</th>
<th>Plasma (μmoles/min/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patient</td>
<td>12/3/77</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>Mother</td>
<td>8.8</td>
<td>1.26</td>
</tr>
<tr>
<td>Father</td>
<td>8.4</td>
<td>1.59</td>
</tr>
<tr>
<td>Controls (n = 9)</td>
<td>11.0–16.4</td>
<td></td>
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</table>

These low plasma levels of ADA activity persist for many weeks after red cell transfusion.

inactivated by prior heating at 80°C for 15 min. Aggregation of normal platelets was unaffected by exogenous ADA.

Nucleotide levels in the patient’s normal platelets are shown in Table 4. There was a twofold to threefold increase in cAMP levels in the patient’s platelets. However, non-cyclic adenine nucleotide levels were normal. Our values for normal subjects agree well with those reported by others, although some authors reported rather high cAMP levels in some normal platelets.

**DISCUSSION**

We have shown that in one patient with SCID and ADA deficiency the platelets are relatively unresponsive to ADP. Schwartz et al. have reported similar findings. However, they also observed a prolonged lag phase and subnormal aggregation responses to collagen at a final concentration of 30 μg/ml, in contrast to the normal responses to collagen in our studies. This discrepancy may be explained by the higher concentration of collagen (1 mg/ml) used by us. However, it is pertinent to emphasize here that the failure of our patient to respond to enzyme replacement by exchange transfusion, in contrast to the success of this therapeutic method in the

**Fig. 3.** Correction of the abnormality of ADP-induced platelet aggregation by purified ADA at all three concentrations of ADP (2.5 μg/ml = 5.0-μM).
ADP (5.0-pM) - ADA + ADA 18 57 (NR 53-102)

Table 3. Correction of Platelet Aggregation Abnormality in ADA-Negative SCID by Exogenous Bovine ADA In Vitro

<table>
<thead>
<tr>
<th></th>
<th>ADP (1.25-µM)</th>
<th>ADP (2.5-µM)</th>
<th>ADP (5.0-µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- ADA</td>
<td>+ ADA</td>
<td>- ADA</td>
</tr>
<tr>
<td>Aggregation rate (ΔO.D./min)</td>
<td>3 22 (NR 22-84)</td>
<td>5 47 (NR 40-108)</td>
<td>18 57 (NR 53-102)</td>
</tr>
<tr>
<td>Maximal aggregation (%ΔO.D.)</td>
<td>3 10 (NR 12-84)</td>
<td>3 20 (NR 20-80)</td>
<td>12 42 (NR 42-87)</td>
</tr>
</tbody>
</table>

*ADA at a final concentration of 2 IU/ml.
NR = normal range.

patient studied by Schwartz et al., may point to some inherent differences in the nature of the enzyme defect in the two cases and may therefore provide a possible alternative explanation for the differences in observations on platelet behavior.

Lack of second-phase platelet aggregation induced by adrenalin in a patient with ADA-negative SCID and in his parents has been reported by Keightley. This abnormality was apparently corrected by incubation with 1-mM uridine for 1 hr at room temperature. However, the second wave of adrenalin-induced aggregation can be absent in 10%-20% of normal subjects. Our patient and his mother did not show this abnormality at the concentration of adrenalin employed.

That the phenomenon of decreased platelet responsiveness to ADP in our patient is related to ADA deficiency is suggested by the remarkable correction of the abnormality by exogenous ADA. Agarwal and Parks observed that the addition of coformycin, a potent ADA inhibitor, to normal PRP significantly prolonged the adenosine inhibition of ADP-induced aggregation of those platelets. A similar prolonged inhibition of ADP-induced aggregation of the platelets in our patient was observed; this was related to the slow clearance of adenosine in his PRP, a consequence of low ADA activity in his plasma and platelets. Schwartz et al., using adenosine at half-molar and equimolar concentrations of ADP, found no further inhibition of the impaired ADP-induced platelet aggregation in their patient. Again, this difference in observation from ours could be due to the higher concentration of adenosine we used (adenosine:ADP at molar ratio of 2.5:1) or to inherent differences in patients.

Adenosine is believed to cause inhibition of platelet aggregation by stimulation of adenyl cyclase, thus raising the level of intracellular cAMP. The increased levels

Table 4. Adenine Nucleotides and cAMP in Platelets of the Patient With ADA-Negative SCID, His Parents, and Controls

<table>
<thead>
<tr>
<th></th>
<th>ATP (µmoles/10^11 platelets)</th>
<th>ADP (µmoles/10^11 platelets)</th>
<th>AMP (µmoles/10^11 platelets)</th>
<th>cAMP (nmoles/10^11 platelets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>5.0</td>
<td>2.0</td>
<td>0.3</td>
<td>3.5</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.5</td>
<td>2.8</td>
<td>0.3</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>8.0</td>
<td>5.5</td>
<td>*</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>5.5</td>
<td>3.1</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
<td>3.0</td>
<td>*</td>
<td>—</td>
</tr>
<tr>
<td>Mother</td>
<td></td>
<td></td>
<td></td>
<td>0.7</td>
</tr>
</tbody>
</table>

*Levels too low for accurate measurement. Dash indicates not determined.
of cAMP in our patient's platelets would support this hypothesis and imply exposure to adenosine in vivo. Grossly elevated levels of cAMP and ATP have been found in the lymphocytes of ADA-negative SCID patients. However, we were unable to document similar increases in ATP levels in the patient's platelets.

Despite the platelet defect in vitro in this patient, he had no bleeding tendencies. The Ivy puncture bleeding time was 4 min (normal range 4–7 min). The patient of Schwartz et al. also had no bleeding manifestations and had a very marginally prolonged Ivy bleeding time. The significance of the in vitro platelet abnormality is therefore unclear.

ACKNOWLEDGMENT

We wish to thank Professor J. Beveridge for allowing us to study this patient.

REFERENCES

In vitro platelet abnormality in adenosine deaminase deficiency and severe combined immunodeficiency

CH Lee, SP Evans, MC Rozenberg, AS Bagnara, JB Ziegler and MB Van der Weyden