Correction by Factor VIII of the Impaired Platelet Adhesion to Subendothelium in von Willebrand Disease

By Harvey J. Weiss, Hans R. Baumgartner, Thomas B. Tschopp, Vincent T. Turitto, and David Cohen

The interaction of platelets with subendothelium has been studied by circulating citrated blood at arterial shear rates through a chamber containing everted segments of rabbit aorta from which the endothelium has been previously removed by balloon catheter. Using this technique, impaired platelet adhesion to subendothelium has been described in patients with von Willebrand disease. The defect was more striking if the vessel segments had been digested with α-chymotrypsin, producing a surface whose platelet reactivity was due primarily to fibrillar collagen. In order to determine whether this defect in platelet adhesion could be corrected by factor VIII, studies were performed in six patients with von Willebrand disease. Cryoprecipitate was chromatographed on Bio-gel A-15m and the following fractions were collected: early-eluting fractions (column buffer), void-volume fractions (factor VIII), and late-eluting fractions which contained the peak absorbance at 280 nm but were devoid of factor VIII activity ("albumin" control). For each of the six patients, perfusion studies were performed on five aliquots of citrated blood to which had been added (1) nothing, (2) column buffer 1, (3) factor VIII, (4) albumin control, or (5) column buffer 2. Adhesion (percent surface coverage) of platelets to α-chymotrypsin digested segments was determined by a morphometric technique. Adhesion values (percent adhesion ± SE) were as follows: (1) 8.2 ± 1.1, (2) 9.8 ± 1.5, (3) 24.7 ± 2.9, (4) 12.5 ± 1.8, and (5) 10.3 ± 1.8 (normal values 28.2 ± 2.1). The corrective effect of factor VIII was more striking in the three patients with the lowest levels of the factor VIII complex. In one patient, platelet adhesion was corrected after transfusion of cryoprecipitate. The finding that factor VIII is required for the adhesion of platelets to subendothelium may be relevant to both hemostasis and to the pathogenesis of atherosclerosis.

Various defects in platelet function, such as impaired retention of platelets in glass-bead filters1 and impaired ristocetin-induced platelet aggregation,2 have been reported in patients with von Willebrand disease.3,4 Recently, we have demonstrated impaired adhesion (but normal aggregation) of von Willebrand platelets to the subendothelial surface of rabbit aorta.5,6 These studies were performed by perfusing citrated blood at arterial shear rates (830 sec⁻¹) through a chamber which contained segments of rabbit aorta from which the endothelium had been previously removed by balloon catheter. We also found that the adhesion defect appeared to be more pronounced with the use of higher citrate concentrations7 or with aortic segments that had been digested with α-chymotrypsin.8 These findings led to the
speculation that an impairment in the adhesion of platelets to components of injured blood vessels may be responsible for the hemostatic defect in von Willebrand disease.5,8

While the above studies indicate that the platelets in patients with von Willebrand disease do not function normally, other studies indicate that this is not due to an intrinsic platelet defect, but rather to the abnormalities of the factor VIII complex which are also characteristic of this disorder. For example, factor VIII (as well as plasma or cryoprecipitate) can correct the abnormalities of platelet retention9,10 and ristocetin-induced platelet aggregation11 in patients with this disorder. The methods that we have used to study the adhesion of platelets in flowing blood to subendothelium appear to approximate more closely the conditions in blood vessels than those which use glass-bead filters or platelet aggregation by ristocetin. Therefore, we have thought it important to see whether the defects in platelet adhesion that we have found in von Willebrand disease can also be corrected by factor VIII.

MATERIALS AND METHODS

Definition and Assays of the Factor VIII Complex

The structure-function relationships which define the procoagulant and platelet function properties of the factor VIII molecule, or molecular complex, remain to be clarified.3,4 In this paper, the antihemophilic factor activity of factor VIII (VIII:AHF) will refer to the plasma activity that corrects the coagulation defect in hemophilia, assayed by a one-stage method.12 The von Willebrand factor activity (VIII:VWF) refers to the activity that supports ristocetin aggregation of washed normal platelets12 and corrects the defect of ristocetin aggregation of patients with von Willebrand disease.11 Factor VIII antigen (VIII:AGN) is the antigen in plasma recognized by a precipitating rabbit antibody to human factor VIII. Levels of VIII:AGN in the plasmas of the patients studied were kindly measured by Dr. Leon Hoyer, using the technique of radioimmunoassay.13

Preparation of Vessel Segments

Everted segments of rabbit aorta, from which the endothelium had been removed by balloon catheter, were prepared as previously described14 and stored at 4°C for up to 4 wk in 0.2 M Tris buffer, pH 7.4, containing 200 Units/ml of penicillin G and 0.2 mg/ml of streptomycin. Enzyme-digested segments were prepared by incubating subendothelial segments 7 mm in length at 37°C for 24 hr in 0.2 M Tris buffer, pH 7.4, which contained 0.4 mg/ml of α-chymotrypsin (approximately 45 U/mg; Worthington Biochemicals, Freehold, N.J.) and 1 mM CaCl2. The digested segments were then washed 10 times with Tris buffer and stored at 4°C in the buffer containing penicillin and streptomycin. Digestion of the vessel segments with α-chymotrypsin removed the amorphous, basement membrane-like material and the microfibrils of elastin present in the subendothelium15 and left a surface whose platelet reactivity was due to the fibrillar collagen that was resistant to enzyme treatment.16

Preparation of Factor VIII Used in Correction Studies

Blood from normal, blood group A, subjects was mixed with 1/10 volume of 3.2% sodium citrate and centrifuged at 2500 g for 15 min to obtain platelet-poor plasma. The plasmas from 11 normal subjects were pooled, frozen at −70°C in aliquots of 13 ml, and then thawed at 4°C for 12 hr. After separating the supernatant by centrifugation, the tubes containing the cryoprecipitate were refrozen at −70°C. On the day of study, 12 tubes of cryoprecipitate (from 156 ml of plasma) were thawed at 37°C for 15 min, pooled, and applied to a glass column 1.6 cm in diameter that had been packed to a height of 20 cm with Bio-gel A-15m (Bio-rad, Richmond, Calif.) and equilibrated with normal saline buffered to pH 7.3 with 10−4 M imidazole.

Elution was performed at room temperature by downward flow under an outlet pressure of 50
Fig. 1. Preparation of factor VIII. Three ml of cryoprecipitate (from 156 ml of pooled normal plasma) were chromatographed on Bio-gel A-15m. A typical elution pattern is shown. One-ml fractions containing either column buffer, factor VIII, or "albumin" were pooled as indicated and used in the correction studies. (The pooled buffer was added to two separate tubes, designated buffer 1 and buffer 2; see Materials and Methods).

cm water (Mariotte Flask); the OD_{280} and VIII:AHF were determined for each of the 1-ml fractions collected. A typical elution pattern is shown in Fig. 1. The following fractions were pooled and used in the correction studies: (1) early fractions, which were devoid of VIII:AHF activity and showed no absorption at 280 nm, were pooled and used as buffer controls (buffer 1 and buffer 2); (2) the fractions in the void volume that showed the highest values for VIII:AHF were pooled and used as the source of factor VIII; and (3) three fractions that eluted later and showed peak absorbance at 280 nm, but lacked VIII:AHF activity, were pooled and served as another control (the "albumin" control). Preparation of the above fractions from the cryoprecipitate took about 2 hr and they were generally ready when the patient arrived for study.

Procurement of Blood From Patients and Preparation of Blood Mixtures Used to Study Platelet Adhesion

Blood from a patient with von Willebrand disease was mixed with 1/10 volume of 108 mM sodium citrate in a plastic flask. The hematocrit was determined and the citrate concentration in the plasma was adjusted to 19.7 mM by further addition of 108 mM sodium citrate. After removing an aliquot (preperfusion specimen) for determination of VIII:AHF and VIII:VWF activity, aliquots of 30 ml were distributed into plastic tubes containing (1) nothing (standard study), (2) 1.5 ml of column buffer (buffer 1), (3) 1.5 ml of factor VIII, (4) 1.5 ml of albumin, or (5) 1.5 ml of column buffer (buffer 2). The mixtures of blood and fractions in tubes 2-5 were incubated for 30 min at 37°C just prior to the perfusion studies described below.

Perfusion Studies and Evaluation of Platelet–Subendothelium Interaction

Each of the above five blood samples was circulated in succession, using the same order 1-5 for each patient, for 10 min at an average flow rate of 160 ml/min (shear rate 830 sec^{-1}) through separate annular perfusion chambers containing three segments of α-chymotrypsin-digested subendothelium. The temperature of the blood and chambers was maintained at 37°C. The flow parameters of the annular chamber and of whole blood perfused at 160 ml/min have been previously reported. Approximately 1 hr elapsed between the first perfusion with the blood in tube 1 (nothing added) and the last perfusion with the second buffer control (tube 5).

Following perfusion, the blood was centrifuged to obtain platelet-rich and platelet-poor plasma, which were used to measure ristocetin-induced platelet aggregation, VIII:AHF, and VIII:VWF. The vessel segments were processed for examination by light microscopy, and the interaction of platelets with the subendothelium was determined by morphometric evaluation of stained cross sections of approximately 0.8 μm thickness. The segments were coded and their origin was not known to the persons doing the evaluations. Approximately 1000 intersection points 10 μm apart were evaluated for each segment and the percent of the surface covered with contact platelets (C) or spread platelets (S) was evaluated as previously described. Platelet adhesion was defined as the surface coverage with platelets (C + S). A platelet thrombus denoted a platelet
Table 1. Patients Studied

<table>
<thead>
<tr>
<th>Patient</th>
<th>Bleeding Time (min)</th>
<th>Hematocrit (%)</th>
<th>Platelet Count ($\times 10^3$/cu mm)</th>
<th>Factor VIII (U/100 ml)</th>
<th>Ristocetin Aggregation (slope $\times 100$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.M.</td>
<td>&gt;60</td>
<td>40</td>
<td>300</td>
<td>&lt;1</td>
<td>&lt;3</td>
</tr>
<tr>
<td>D.R.</td>
<td>&gt;60</td>
<td>37</td>
<td>276</td>
<td>&lt;1</td>
<td>&lt;3</td>
</tr>
<tr>
<td>V.M.</td>
<td>&gt;60</td>
<td>42</td>
<td>190</td>
<td>&lt;1</td>
<td>&lt;3</td>
</tr>
<tr>
<td>J.L.</td>
<td>40</td>
<td>36</td>
<td>206</td>
<td>42</td>
<td>4</td>
</tr>
<tr>
<td>A.S.</td>
<td>28</td>
<td>37</td>
<td>212</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>P.H.</td>
<td>20</td>
<td>35</td>
<td>138</td>
<td>14</td>
<td>3</td>
</tr>
</tbody>
</table>

VIII:AHF, VIII:VWF, hematocrit, and platelet count values were obtained on the day of study. Other values were obtained on previous occasions; some of these have been reported previously.1,23

aggregate 5 $\mu$m or more in height. T signified surface coverage with such thrombi (aggregates) and 100 T/S expressed such thrombi as a percentage of the surface already covered with spread platelets.

Patients
We studied the six patients with von Willebrand disease shown in Table 1. In three patients (C.M., D.R., and V.M.), the levels of all three components of the factor VIII complex were below the limits that could be detected by the methods used (VIII:AHF < 1, VIII:VWF < 3, and VIII:AGN < 0.3 U/100 ml) and the Ivy bleeding time in these patients with severe von Willebrand disease exceeded 60 min. In the other three patients (A.S., J.L., and P.H.), the laboratory values were less strikingly abnormal and measurable values of the factor VIII complex were obtained. Two of the patients (A.S. and J.L.) have been reported elsewhere to have a "variant" form of von Willebrand disease characterized by an increase in the ratio of VIII:AHF to VIII VWF, an increased lability of VIII:AHF activity at 37°C, and an increased mobility of VIII:AGN on crossed immunoelectrophoresis.20 All patients were blood group O except patient P.H., who was group A.

Analysis of Results
To summarize the design of the experiment, 150 ml of citrated blood from each patient was distributed into five tubes containing either nothing (standard study), or test substances (buffer I, factor VIII, albumin, buffer 2) prepared the same day from pooled cryoprecipitate. Each of these five blood samples was circulated through a separate chamber containing three vessel segments; platelet adhesion was calculated by averaging the adhesion values for the three segments. To determine whether platelet adhesion in the six patients was modified by any of the test substances, we used analysis of variance and the technique of subsidiary analysis described by Anderson and Bancroft for a similar model.21

Transfusion Studies
Patient V.M. received cryoprecipitate for treatment of gastrointestinal bleeding on two occasions 2 mo apart.

RESULTS

Untreated Blood
In normal subjects 28.2% $\pm$ 2.1% (SE) of the $\alpha$-chymotrypsin–digested subendothelial surface is covered with adherent platelets after the 10-min perfusion period.4 Similar to findings previously reported,5 platelet adhesion was decreased in the untreated blood of all patients studied (Figs. 2 and 4, nothing
added). The average value for platelet adhesion in the untreated blood samples was 8.2% ± 1.1%.

**Effect of Factor VIII and Control Fractions in Individual Patients**

*Patients with "severe" von Willebrand disease.* The effect of adding various fractions to the blood of patients C.M., D.R., and V.M. with severe von Willebrand disease is shown in Fig. 2. When compared with the untreated sample (nothing added), buffer 1, albumin, or buffer 2 had no effect on platelet adhesion. In all cases, adhesion values remained low and were similar to those obtained on untreated blood. In contrast, adhesion was markedly increased by factor VIII (Fig. 2, solid bars) in all patients. Figure 3 is a photomicrograph showing the corrective effect of factor VIII on platelet interaction with the collagen fibrils of α-chymotrypsin–digested subendothelium. The addition of factor VIII also corrected the defects in ristocetin-induced platelet aggregation and increased the levels of VIII:AHF (46–68 U/100 ml) and VIII:VWF (105–140 U/100 ml).

*Patients with "mild" or "variant" von Willebrand disease.* The results obtained on the three patients with mild or variant von Willebrand disease are shown in Fig. 4. In patients J.L. and A.S., platelet adhesion values in blood that had been incubated with factor VIII were, with one exception (J.L. buffer 2), greater than any of the various control values. However, the differences between factor VIII–treated and control blood samples were less striking than in the patients with severe von Willebrand disease. The corrective effects of factor VIII on ristocetin aggregation, VIII:AHF (57–70 U/100 ml) and VIII:VWF

![Fig. 2. Studies on patients with "severe" von Willebrand disease. Results depict postperfusion values of platelet adhesion, ristocetin-induced platelet aggregation (RIPA), and factor VIII on untreated blood (nothing added) and blood samples to which various fractions had been added. Brackets depict the SEM. The preperfusion (PRE) factor VIII values are also shown. (See Table 1 for normal values and other details on these three patients.)](image-url)
Fig. 3. Interaction of platelets with the fibrillar collagen of α-chymotrypsin-digested subendothelium. Representative findings in patient C.M., showing the interaction of platelets with α-chymotrypsin-digested subendothelium after perfusion of vessel segments with blood to which either buffer (A) or factor VIII (B) had been added. Bar indicates 10 μm.

(135–150 U/100 ml) were again seen. The results obtained on the five blood samples of P.H. were difficult to interpret, but did not suggest any significant effect of factor VIII on platelet adhesion. Of interest, ristocetin-induced platelet aggregation remained markedly abnormal in his factor VIII–treated blood, despite an increase in the level of VIII:VWF to 91 U/100 ml.

As seen in Fig. 4, the previously reported2 lability of VIII:AHF in patients A.S. and J.L. was again observed. For example, after incubation and perfusion,

Fig. 4. Studies on patients with “mild” or “variant” von Willebrand disease. See Fig. 2 legend.
the VIII:AHF level in the four control blood samples of patient J.L. decreased from 42 U/100 ml to values of 12–15 U/100 ml.

Overall Group Effects of Various Treatments on Platelet Adhesion

For each of the six patients, there were five treatments (for purposes of analysis, the untreated sample to which nothing was added was also considered a treatment) and triplicate values (one for each vessel segment) were obtained for each treatment.

Mean values for each treatment. The means of the 18 values in each of the 5 treatment groups are shown in Fig. 5. The mean value of 24.7% ± 2.9% (SE) for the factor VIII–treated blood was significantly greater (p < 0.05) than that of any of the other treatments and was similar to the value of 28.2% ± 2.1% obtained for normal subjects in previous studies.6

Analysis of variance. The analysis of variance results are shown in Table 2. When the triplicate values (residual) were used as the error term, it was apparent that there were differences between the patients (p < 0.001) and marked differences (F = 22.32, p < 0.0001) between treatment groups. The interaction term (patients × treatments) was also significant (p < 0.001). However, even when the latter was used as the error term, the differences between the treatment groups were still highly significant (p < 0.005).

Table 2. Analysis of Variance

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>Degrees of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Residual</td>
<td>Interaction</td>
</tr>
<tr>
<td>Patients</td>
<td>5</td>
<td>1156.3</td>
<td>231.2</td>
<td>6.46*</td>
</tr>
<tr>
<td>Treatments</td>
<td>4</td>
<td>3194.9</td>
<td>798.7</td>
<td>22.32†</td>
</tr>
<tr>
<td>Interaction</td>
<td>20</td>
<td>2333.4</td>
<td>116.7</td>
<td>3.26*</td>
</tr>
<tr>
<td>Residual</td>
<td>60</td>
<td>2147.1</td>
<td>35.8</td>
<td>—</td>
</tr>
</tbody>
</table>

The results are shown for the six patients and five treatments (nothing, buffer 1, factor VIII, albumin, and buffer 2). Three values (one for each of the three vessel segments in the perfusion chamber) were obtained for each treatment in each patient, accounting for 90 observations and a total of 89 degrees of freedom.

*p < 0.001.
†p < 0.0001.
‡p < 0.005.
**Subsidiary analysis.** We tested the hypothesis that treatment with factor VIII was significantly different from the other four treatments by using the method of Anderson and Bancroft for a similar model,\(^2\) as described in the Appendix. This analysis showed that treatment with factor VIII was significantly different from the other treatments (\(p < 0.001\)). In addition, there were no significant differences among the other treatments (Appendix).

**Transfusion Studies**

The results obtained after transfusing patient V.M. with cryoprecipitate are shown in Fig. 6. They may be summarized as follows: adhesion values were increased 2 hr after transfusion (on one occasion to a normal value) and then decreased during the next 6–48 hr. The increased adhesion values were always associated with some shortening (but never normalization) of the bleeding time. The abnormal ristocetin-induced platelet aggregation was never completely corrected, although on one occasion the level of VIII:VWF increased to 120 U/100 ml.

**Platelet Aggregation (Thrombi)**

Factor VIII had no effect on platelet aggregation. Values for 100 T/S were as follows (mean ± SE): untreated, 55.8 ± 5.4; buffer 1, 48.5 ± 5.5; factor VIII, 49.2 ± 4.1; albumin, 40.3 ± 5.6; buffer 2, 53.0 ± 5.9.

![Graph of Platelet Aggregation](image)
DISCUSSION

The arrest of bleeding from transected blood vessel is due, in part, to the formation of platelet plugs at the end of these vessels. Light microscopic examination of bleeding time wounds has shown impaired platelet plug formation in patients with von Willebrand disease. When examined by electron microscopy, the lips of transected vessels in patients with this disorder are found to be devoid of platelets in the area of the endothelial cells and also in deeper areas of the vessel wall in which platelets are normally seen in close proximity to connective tissue.

Because of the problems involved in quantifying the interaction of platelets with the vessel wall in vivo, we used an in vitro model in which both platelet adhesion and aggregation on subendothelium could be evaluated using citrated blood and flow conditions similar to those in large arteries. As in previous studies, we found that platelet adhesion was significantly decreased in six patients with von Willebrand disease. Addition of factor VIII to the blood before perfusion corrected the defect in platelet adhesion to the collagen fibrils of α-chymotrypsin-digested subendothelium. This finding, together with the report of Hovig and Stormorken (who have found that platelet plugs are frequently seen after transfusion of plasma), provides direct evidence that factor VIII may be involved in the adhesion of platelets to the vessel wall. Hence, the deficiency of factor VIII seen in von Willebrand disease could explain why these patients have a long bleeding time. It is not clear why the corrective effects of factor VIII in vitro were more apparent in the three patients with “severe” von Willebrand disease than in the three patients with “variant” or “mild” forms of this disorder. Since the blood samples were kept at room temperature for a period of 1–2 hr before testing, it is possible that variant factor VIII:VWF in the platelets of these patients could have affected the results.

The mechanism by which factor VIII promotes platelet adhesion to the vessel wall remains to be determined. In the present study, we have studied platelet adhesion to vessel segments that had been digested with α-chymotrypsin. Since fibrillar collagen is the main platelet-reactive material in these digested vessels, the findings in the present study suggest that factor VIII can be involved in the adhesion of platelets to collagen. For example, a number of glycoprotein cell-attachment factors have now been described, including one that may be specific for the attachment of some cells to collagen. Hence, factor VIII, a glycoprotein, could be another example of such a cell-attachment factor.

At present, however, we are reluctant to suggest that factor VIII specifically promotes the adhesion of platelets to collagen. For example, in previous studies we have suggested that the impairment of platelet adhesion in von Willebrand disease is due to a defect in the initial attachment (contact) of platelets to the subendothelial surface; the ability of contacted platelets to spread upon the surface is normal. Previous studies have shown that the initial attachment (as opposed to spreading) of a platelet to a surface may be independent of the surface. In addition, factor VIII bound to platelets appears to mediate platelet-to-platelet adhesion (e.g., aggregation) induced by ristocetin. For these reasons, we are hesitant to suggest that the impairment of platelet ad-
hesion that we have found to be correctable by factor VIII in von Willebrand disease is due to a specific defect in the adhesion of platelets to collagen. Thus, the platelet-adhesion defect in von Willebrand disease could be more general, and the mechanism by which factor VIII promotes platelet adhesion requires further clarification.

Although the studies reported herein, as well as those of Hovig and Stormorken, suggest that the hemostatic defect in von Willebrand disease could be due to the impaired adhesion of platelets to (nonendothelial) elements of the vessel wall, endothelial-mediated abnormalities could also be involved. Previous studies have established that factor VIII is present and synthesized in endothelial cells and it has been suggested that endothelial cell factor VIII may play a role in hemostasis. For example, the degree to which the bleeding time is corrected after transfusion does not always correlate with the plasma level of factor VIII:VWF (measured by the ristocetin method), and in our limited transfusion studies the bleeding time was not normalized even with complete correction of the platelet-adhesion defect (Fig. 5). In addition, endothelial cell factor VIII remains undetectable by immunofluorescent techniques after transfusion of factor VIII to patients with von Willebrand disease. Thus, it is entirely possible that interaction of platelets with both injured endothelial cells and elements of the vessel wall could play an important role in hemostasis.

Finally, platelet-induced vessel injury, possibly through release of a smooth muscle mitogenic factor, has been proposed as an important initiating mechanism in the pathogenesis of atherosclerosis. The findings reported previously and herein that the adhesion of platelets to subendothelium is decreased in von Willebrand disease could account for the observation that pigs with this disorder are less prone to develop atherosclerosis than are normal pigs. Hence, the findings that factor VIII is involved in the adhesion of platelets to the vessel wall could have important implications regarding the pathogenesis of atherosclerosis.

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APPENDIX

Analysis of Variance: Subsidiary Analysis

The five treatment groups—nothing (N), buffer 1 (B1), factor VIII (VIII), albumin (Alb), and buffer 2 (B2)—are treated as columns, the patients as rows. We wish to test the hypothesis that treatment of blood with factor VIII is significantly different from the other treatments. Using the method described by Anderson and Bancroft, we will test whether the sum of all 18 measurements in column VIII (6 patients x 3 replicates) is significantly different from the sum of measurements in the other columns.

Since we are interested in testing column VIII against the other columns collectively, we need a variable which characterizes the sum of measurements in these columns collectively. The most straightforward procedure would be to average the column totals and test the average column total thus obtained against the column VIII total. An equivalent, but computationally more con-
FACTOR VIII AND PLATELET ADHESION

A convenient procedure, is to test four times the column total of column VIII against the sum of the four other column totals. This procedure gives us a linear form (1):

\[ I = N + B_1 + Alb + B_2 - 4 \cdot VIII \]

where \( N \) is the sum of all measurements in column \( N \), \( B_1 \) the sum of all measurements in column \( B_1 \), \( Alb \) the sum of all measurements in column \( Alb \), \( B_2 \) the sum of all measurements in column \( B_2 \), and \( VIII \) the sum of all measurements in column \( VIII \).

Under the null hypothesis the expected values of all column totals are equal. Hence the expected value of \( I \) is zero.

The observed value of \( I \) is

\[ I = 147.3 + 176.0 + 225.8 + 185.1 - 1776.8 = -1042.6. \]

To estimate the variance of \( I \) we use

\[ V\left( \sum_{i=1}^{N} a_i x \right) = \sum_{i=1}^{N} a_i^2 V(x). \] (1)

The column total, in each case, is the sum of 18 measurements; hence each column’s variance equals \( 18 \sigma^2 \), where \( \sigma^2 \) is the variance in a single measurement. This gives

\[ V(I) = V(N) + V(B_1) + V(Alb) + V(B_2) + 16V(VIII). \] (2)

Using (2), we have

\[ V(I) = 18 \sigma^2 + 18 \sigma^2 + 18 \sigma^2 + 18 \sigma^2 + 16 \cdot 18 \sigma^2 \]
\[ = 360 \sigma^2. \] (3)

Finally, using our estimate \( \sigma^2 = 35.8 \) (Table 2), we have \( V(I) = 12882.6 \) and \( \sigma(I) = \sqrt{V(I)} = 113.5. \)

We may now form the \( t \) statistic

\[ t = \frac{-1042.6}{113.5} = 9.19. \]

From a table of the \( t \) statistic, we see that the probability of the null hypothesis \( p(H_0) \) is <0.001.

There is no a priori reason to single out column \( VIII \) for special consideration. In fact, there are five ways to choose one “special” column from a group of five. Therefore, we can increase our estimate of the probability of the observed outcome under the null hypothesis by

\[ p = 5 \ p(H_0) < 0.005. \]

Since \( p \) is very small, we reject the null hypothesis and say that treatment \( VIII \) is significantly different from the others.

Furthermore, the sum of squares for \( I \) (there is only one term, of course) is \((-1042.6)^2 \). By (3), an estimate for total sum of squares is \((\text{sum of squares for } I)/360 = (-1042.6)^2/360 = 3019.5 \). However, the total sum of squares for the treatment was 3194.9 (Table 2). Therefore, the other four treatments combine to contribute only 175.4 toward the total sum of squares. We therefore say that no other treatment is significantly different from the rest.

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