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Evidence that Factor VIII and the Ristocetin Aggregating Factor ($VIII_{Rist}$) are Separate Molecular Entities[1] (38280)

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The most highly purified preparations of human factor VIII have at least three clearly defined properties. They shorten the clotting time of hemophilic plasma, an activity which will for convenience be referred to as $VIII_{coag}$; they give a precipitin reaction with a rabbit antibody prepared against the preparation, a property referred to as the factor VIII related-antigen ($VIII_{R-Ag}$); and in the presence of the antibiotic, Ristocetin, they correct the defective aggregation of platelets found in von Willebrand's disease (1–4), an activity which will be referred to as $VIII_{Rist}$. There are two prevailing concepts: (a) that each property represents a function of the same molecule, (b) that there is more than one molecule, each with one or more properties (5). The purpose of the experiments described below was to determine whether $VIII_{coag}$, $VIII_{Rist}$, and $VIII_{R-Ag}$ reside on the same or on different molecules. The addition of a naturally occurring human factor VIII antibody known to be of the IgG class neutralizes $VIII_{coag}$ without affecting the expression of $VIII_{Rist}$ or $VIII_{R-Ag}$ (3). Rabbit antibodies, on the other hand, prepared against highly purified factor VIII preparations neutralize $VIII_{Rist}$ and $VIII_{R-Ag}$ (3), but their $VIII_{coag}$ neutralizing activity may be relatively low (6, 7). These findings are not inconsistent with the one-molecule hypothesis as the antibody could neutralize one side chain carrying an active site without affecting others. If the one-molecule hypothesis is correct, following the neutralization of $VIII_{coag}$ or $VIII_{Rist}$ by the appropriate antibody, the addition of a second precipitating antibody directed against the IgG class should precipitate both $VIII_{coag}$ and $VIII_{Rist}$ activities bound to the first antibody; as the factor VIII molecule would carry all the active sites, the supernatant will be devoid of all the remaining properties of the molecule. With the multiple-molecule hypothesis, only the neutralized activity should be precipitated while the other activity remains in the supernatant.

Materials and Methods. The human factor VIII used as antigen was a vial of lyophilized cryoprecipitate (Courtland) containing approximately 250 U. After reconstitution of the vial contents in 25 ml 0.1 *M* saline, the pH was adjusted to pH 6.1 with 1 *M* citric acid and this was followed by a differential polyethylene glycol (6000) precipitation step (8). The 3–10% precipitate was resuspended in 1.5 ml of 0.25 *M* NaCl, 0.025 *M* imidazole, and 0.1% Na azide, pH 6.5. This solution, which contained 190 U $VIII_{coag}$, was applied to a 1.0 × 86 cm Sepharose 4B column. The peak tubes (8 U/ml, 3,000-fold pure) of the void-volume material were pooled and frozen in five 1-ml aliquots.

New Zealand white female rabbits were immunized using the following regimen: 1 ml alumina C γ-Gel (A Grade, Calbiochem) was mixed with a 1 ml aliquot of the thawed-out factor VIII and injected into two subcutaneous sites along the back. These injections were repeated four times at weekly intervals. The serum obtained 1 week after the last injection was adsorbed twice with $Al(OH)_3$ and heated for 1 hr at 56° to remove all traces of thrombin; normal rabbit serum treated similarly was used as the control. The antiserum was then adsorbed with 1/20 volume of plasma from a patient with severe von Willebrand's disease. The adsorbed antiserum and control serum were then precipitated by sodium sulfate (0 – 18%) and the crude IgG precipitates redissolved in one-fourth of the original serum volume of 0.14 *M* NaCl, pH 7.2,

and dialyzed overnight against 0.14 *M* NaCl. The IgG prepared from the antiserum gave a single precipitin arc on immunoelectrophoresis against normal cryoprecipitate but not against the cryoprecipitate of three patients with von Willebrand's disease. When added to normal plasma in an amount sufficient to neutralize 95% of $VIII_{R-Ag}$ no significant reduction of factor $VIII_{coag}$ was observed. However, in higher concentrations, factor $VIII_{coag}$ inhibition could be achieved. The antiserum therefore was not monospecific and does not differ qualitatively from rabbit factor VIII antibodies used by others.

Antibody against $VIII_{coag}$ was derived from plasma from a hemophilic patient with an inhibitor titer of 40 U/ml. This and a normal plasma control were treated in a similar manner to the rabbit serum and precipitated by sodium sulfate as described above.

Platelet suspensions were prepared by the method of Walsh (9) from normal citrated plasma but the platelets were washed four times and the albumin cushion in the last wash was reduced to 0.5 ml. The suspension contained between 300,000 and 500,000 platelets per mm^3.

Ristocetin aggregation was determined by placing in a well of a microtiter plate, 10 μl platelet suspension, 50 μl saline, 20 μl of the test material, 10 μl of plasma from a patient with very severe von Willebrand's disease (less than 1% factor VIII and no detectable $VIII_{R-Ag}$, or $VIII_{Rist}$), and 10μl Ristocetin (final concentration 1mg/ml) in this order. The mixture was gently agitated for 5 min. Serial dilutions of the test material were tested and the highest dilution producing unequivocal aggregation determined using a hand lens. Normal plasma usually gave a titer of 1:10 and the cryoprecipitate (20 U $VIII_{coag}$/ml) a titer of 1:250.

The crude IgG preparations from the naturally occurring human anti-$VIII_{coag}$, a normal human control, the rabbit antibody, and a normal rabbit control were purified by ion exchange chromatography on QAE Sephadex A-50 (Pharmacia) (10). The four purified IgG preparations (4 mg of each) were labeled with I^{125} by the method of Helmkamp (11).

$VIII_{coag}$ was assayed by a one-stage technique of Langdell *et al.* (12) using human hemophilic plasma as substrate and kaolin as the activator. This method cannot discriminate between values lower than 5% and the standard deviation at the 100% value is ±16%.

$VIII_{R-Ag}$ was determined by the method of Zimmerman *et al.* (13). Using this technique, we obtain no rocket when normal plasma is diluted 1 in 16 while a 1 in 128 dilution of the cryoprecipitate used in these experiments was the highest titer at which a rocket was detectable.

Goat anti-rabbit IgG and goat anti-human IgG antisera were precipitating antibodies purchased from Calbiochem. They were adsorbed twice with Al $(OH)_3$ and heated at 56° for 1 hr.

No thrombin, $VIII_{coag}$, $VIII_{Rist}$, or $VIII_{R-Ag}$ activities were found in any of the treated antisera or the control sera used in these studies.

Results. To 0.5 ml cryoprecipitate was added 0.2 ml of the crude IgG preparation from the patient with the $VIII_{coag}$ antibody and 10 μl I^{125} labeled IgG from the same patient. After 30-min incubation, it was found (Table I) that $VIII_{coag}$ was less than 5% while the $VIII_{R-Ag}$ and $VIII_{Rist}$ activities did not differ significantly from those of the control (a similar mixture except that both the radioactive tracer and unlabeled crude IgG were derived from a normal control subject). To

TABLE I. Effect of Human Naturally Occurring Factor $VIII_{coag}$ Antibody on Factor VIII[a].

Incubation mixture	Type of assay	After 30-min incubation	After addition of second antibody
		Test control	Test control
0.5 ml cryoprecipitate	$VIII_{coag}$	< 5 (100)	< 5 (90)
0.2 ml human anti-factor	$VIII_{R-Ag}$	100 (100)	95 (90)
$VIII_{coag}$ or normal IgG	$VIII_{Rist}$	+ (+)	+ (+)

[a] 10 μl of I^{125} labeled IgG from the patient with the anti-$VIII_{coag}$ or from a normal subject were included in the test or control mixtures, respectively. $VIII_{coag}$ and $VIII_{R-Ag}$ are recorded in percentage of values of the control mixture. The values of the normal control are shown in parenthesis. $VIII_{Rist}$ assays are recorded as positive or negative at a 1 in 16 dilution for the control and undiluted for the test.

a 0.5-ml aliquot of the test mixture was then added a volume of goat anti-human IgG sufficient to precipitate most of the counts in the incubation mixture. The mixture was centrifuged at 2000 rpm for 10 min and the radioactive counts in the supernatant determined using a well-type gamma scintillation counter. 0.5 ml of goat anti-human IgG was required to produce maximum precipitation (96%). The same amount of goat anti-human IgG was added to the control tube resulting in precipitation of 90% of the counts. The concentration of $VIII_{Rist}$ and $VIII_{R-Ag}$ in the supernatant of the mixture containing the human anti-$VIII_{coag}$ was not significantly different from that of the control and, taking into account the dilution factor, not significantly altered by the addition of the second antibody. However, unlike the control, it had no measurable $VIII_{coag}$ activity.

The same experiment was now repeated substituting rabbit IgG for human IgG preparations. Thirty minutes following the addition of the first antibody, $VIII_{coag}$ did not significantly differ from that of the control (Table II) while there was no Ristocetin aggregation even when the test material was used undiluted. To a 0.5-ml aliquot of incubation mixture was added 0.3 ml of goat anti-rabbit IgG to attain equivalence; and the same amount of the antibody was added to the control. The two mixtures were then centrifuged (2000 rpm for 10 min). The precipitate from the tube with the rabbit anti-VIII contained 95% of the total radioactivity and the control 87%. The supernatants were assayed and a small, but possibly insignificant, reduction in the $VIII_{coag}$ in both mixtures and a small decrease in $VIII_{R-Ag}$ in the control were found but no change in $VIII_{Rist}$. The supernatant from the mixture containing the rabbit anti-VIII retained most of the $VIII_{coag}$ activity while there was no detectable $VIII_{Rist}$ or $VIII_{R-Ag}$.

Discussion. The finding that $VIII_{coag}$ can be separated from $VIII_{Rist}$ and $VIII_{R-Ag}$ using a double-antibody technique is consistent with the multiple- but not with one-molecule hypothesis. In the experiments using human IgG, although 96% of the total counts from the labeled IgG were incorporated into the precipitate, it could be argued that the soluble antibody–antigen complexes selectively failed to be precipitated by the second antibody. The same arguments could also apply to the second type of experiment using the rabbit factor VIII antibodies, but is somewhat weakened by the fact that these antibodies, unlike the naturally occurring human antibodies, tend to be precipitating. For the one-molecule hypothesis to be valid, it is necessary to postulate selective precipitation in both types of experiments although the antibodies show several differences. Therefore, while not conclusive, the results support the multiple-molecule hypothesis. This work confirms and extends previous observations which indicated that factor $VIII_{coag}$ and $VIII_{R-Ag}$ were distinct molecular entities (6, 14). Furthermore, Weiss and Hoyer (15) have shown that $VIII_{Rist}$ remains with $VIII_{R-Ag}$ in the void volume upon gel filtration of human cryoprecipitate in 0.8 *M* NaCl, while under these conditions $VIII_{coag}$ shifts to a lower molecular weight form. Brown *et al.* (16) have recently shown that highly purified bovine factor VIII prepared by the method of Schmer *et al.* (17) will aggregate bovine platelets in the presence of Ristocetin. They were able to separate the $VIII_{coag}$ from the $VIII_{Rist}$ by QAE Sephadex A-25 ion exchange chromatography. The $VIII_{Rist}$ appeared in the major protein peak together with the $VIII_{R-Ag}$ while the $VIII_{coag}$ eluted later after most of the protein had been eluted from the column.

No conclusions can be drawn from our work as to whether $VIII_{Rist}$ and $VIII_{R-Ag}$ are discrete or the same molecular entities.

TABLE II. Effect of Rabbit Factor VIII Antibody on Factor VIII[a].

Incubation mixture	Type of assay	After 30-min incubation	After addition of second antibody
		Test control	Test control
0.5 ml cryoprecipitate	$VIII_{coag}$	108% (100)	90% (94)
0.2 ml rabbit anti-VIII	$VIII_{R-Ag}$	<3% (100)	<3% (86)
or normal IgG	$VIII_{Rist}$	— (+)	—(+)

[a] 10 μl of I^{125}-labeled IgG from a rabbit factor VIII antiserum or normal rabbit serum were included in the test or control mixture, respectively. The results are recorded in the same manner as in Table I.

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