Studies of the Antihemophilic Factor (AHF, Factor VIII) Produced in von Willebrand's Disease.* (29030)

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In 1959 one of us pointed out that the apparent dependence of plasma antihemophilic factor (AHF, F. VIII) level on the activity of 2 genetic loci must have significance with respect to AHF synthesis(1). At that time only structural genes were generally considered operative in protein synthesis. This coupled with the widespread acceptance of the "one gene—one enzyme" concept impeded consideration of models for AHF synthesis involving 2 loci. Since then it has become apparent that both structural and regulatory genes function in bacteria(2) and that 2, non-allelic structural genes are sometimes involved in protein synthesis in mammals, *e.g.*, hemoglobin in humans.

It has been found experimentally that the 2 mutant phenotypes showing reduced plasma AHF levels, hemophilia A and von Willebrand's disease (v.W.d.), do not "complement" *in vitro* as do hemophilias A and B, for example. This is in fact the basis for the assertion that AHF is "deficient" in v.W.d. A paradoxical type of "complementation" does occur *in vivo,* however. Plasma from persons with autosomal von Willebrand's disease is ineffective when transfused into persons having X-linked hemophilia A(3), but the reciprocal transfusion has a striking effect. When plasma from a person with X-linked hemophilia A and low AHF is transfused into a person with v.W.d. with low plasma AHF, large amounts of AHF appear in the blood(4). The quantity of AHF appearing is so large that it has been interpreted as new synthesis, and the observation has been confirmed in several laboratories(5,6). This finding has led to a considerable elaboration of the possible models of AHF synthesis. The locus on the X-chromosome has been suggested as a structural locus coding for the peptide sequence of AHF, while the locus on the (as yet unidentified) autosomal chromosome has been suggested as regulatory(7,8).

This complex model of AHF synthesis has at least one consequence which can be tested experimentally. If the autosomal (v.W.d.) locus is regulatory, the AHF produced in the person with v.W.d. after a transfusion of hemophilia A plasma should be structurally normal. This paper describes experiments carried out to examine the AHF appearing under these conditions. It will be shown that this AHF was not distinguished from that of normal persons by 3 independent tests.

Methods and materials. All AHF assays were performed by the method of Langdell *et al*(9) modified as follows: Canine hemophilic substrate plasma was used(10); this was activated with 5 mg kaolin/ml plasma (11,12) at 28°C for 10 minutes, then diluted and placed in an ice bath. The thromboplastin generation tests were performed according to Biggs and Douglas(13). To ensure that differences in AHF levels between the plasmas would not influence the rate or amount of thromboplastin generated, all of the plasmas were first diluted with normal saline to 32% AHF, the lowest AHF level observed by the Langdell method in a person with v.W.d. after transfusion.

Plasma was obtained from 3 unrelated men with severe sex-linked hemophilia A and one normal person for compatible transfusion into 4 persons with von Willebrand's disease belonging to 3 unrelated families. The amounts transfused ranged from 6.7 to 9.7 ml/kg body weight. After the plasma was administered, blood samples were withdrawn from the recipients at 6 hour intervals for assay of AHF. When the recipients' plasmas showed an apparent peak level of AHF activity, each person was bled to obtain one 250 ml unit of plasma. The plasma was stored in 10 ml aliquots at —20°C until used for preparation of AHF-rich Fraction I-O(14), or for temperature and pH stability studies. Plasmas

* Supported in part by USPHS grants.

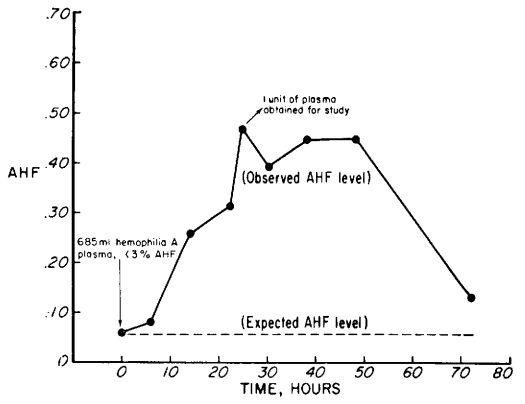


FIG. 1. Expected and observed AHF levels in a patient with von Willebrand's disease after receiving 9.7 ml/kg body wt of fresh-frozen hemophilia A plasma containing no measurable AHF activity. Note prolonged period of increased AHF activity (>50 hr).

from normal subjects were stored and tested under the same conditions in parallel tests.

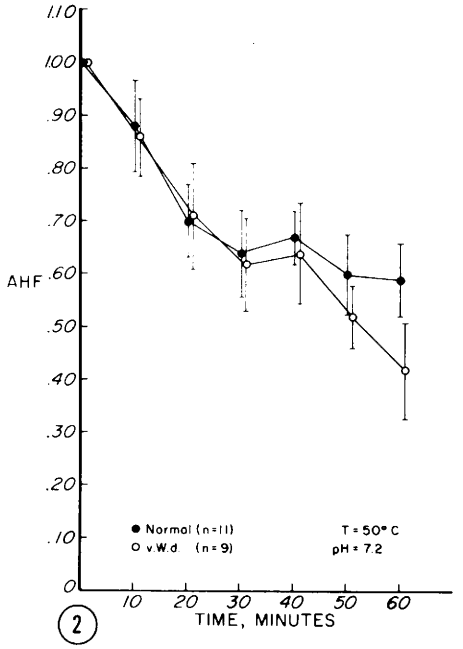
Results: Transfusion of v.W.d. recipients with hemophilia A plasma. The result of transfusing a woman having v.W.d. with plasma from a man with sex-linked AHF deficiency is shown in Fig. 1. This type of increase in AHF was obtained on the 3 persons with v.W.d. receiving compatible plasma from persons with hemophilia A. The fourth person with v.W.d. was transfused with normal plasma and gave the usual response to a transfusion of compatible normal plasma(5), *i.e.*, a larger and more prolonged rise in AHF than is observed in patients with hemophilia A with the same amount of plasma.

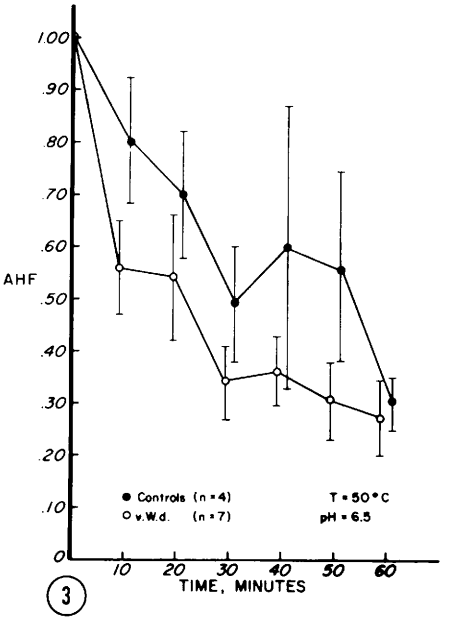
Effect of heat on the newly appearing AHF. The denaturation at 50°C of the AHF in normal control plasma and in plasma from the transfused v.W.d. patients is shown in Fig. 2. The disappearance rates of AHF from the 2 types of plasma are seen to be not different. Fig. 3 shows the AHF loss after heating the low temperature-ethanol Fraction I-O prepared from the 2 types of plasma. The fractions were quite unstable and any real difference is masked by the large standard errors.

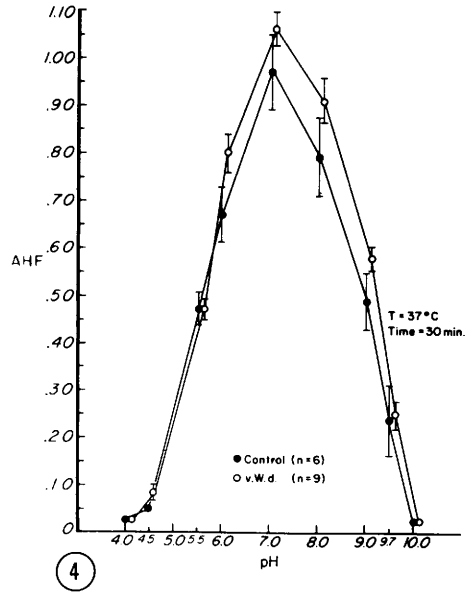
Effect of pH on the newly appearing AHF. The effect of pH on the AHF of the transfused persons with v.W.d. is shown in Fig. 4. No difference is seen between the types of AHF.

Efficiency of newly appearing AHF in thromboplastin generation. The plasmas from 3 persons with v.W.d. obtained after transfusion with hemophilia A plasma and one after normal plasma were compared with normal plasma and an untransfused v.W.d. in the thromboplastin generation test. It can be seen (Fig. 5) that the AHF in v.W.d. forms thromboplastin as rapidly and to the same degree as the normal control.

Discussion. It has been confirmed that persons with autosomal AHF deficiency (von Willebrand's disease) produce large quantities of AHF when transfused with AHF deficient plasma from persons with hemophilia A. This occurs despite the fact that "complementation" does not occur *in vitro*. One prediction from a model suggesting that the X-locus for AHF is structural while the autosomal locus is regulatory is that the AHF which appears in v.W.d. after transfusion will be structurally normal. The AHF obtained under these conditions has been tested by 3 methods and the results fulfill the prediction. This *may* imply that the model is correct. On the other hand, the prediction may have been fulfilled for other reasons. Perhaps the wrong parameters were examined, or the large standard errors of the tests masked a real difference. Further tests are obviously needed before one can feel reasonably certain that mutation of a regulatory gene is the basis of v.W.d. An examination of persons with sex-linked and autosomal AHF deficiency using an antibody prepared against wild type AHF might be very illuminating. Also, transfusion studies should be carried out on persons homozygous for von Willebrand's disease if such persons can be found. Failure to obtain "new synthesis" in a *homo*zygote would throw great doubt on a model which implies that the reduced AHF levels in v.W.d. heterozygotes is due entirely to reduction in an effector substance which neutralizes the repressor of an X-chromosome operon directly coding the AHF molecule(7,8).







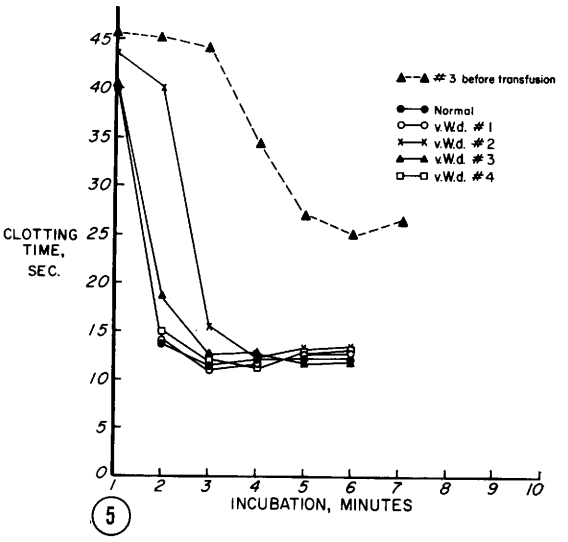


FIG. 2. Loss of AHF activity in normal plasmas and von Willebrand's disease plasmas at 50°C. Patients' plasmas were obtained at peak AHF levels. Plasma from each of 3 recipients was tested 3 times (n = 9). Each plasma was thawed, adsorbed with $Al(OH)_3$, adjusted to pH 7.2 by diluting to 80% concentration with imidazole buffer(15) and placed in a 50°C constant temperature water bath. As soon as temperature of the plasma reached 50°, 1.0 ml was removed for the starting control and its AHF level recorded as 1.00. At subsequent 10 min intervals, 1.0 ml samples were removed from the water bath, cooled in ice and centrifuged at 3,000 rpm to remove heat-precipitated proteins; the samples were accumulated in an ice bath at 0°C until the whole group could be assayed for AHF activity. Points represent mean values and vertical lines a single standard error of mean.

FIG. 3. Loss of AHF activity in Fraction I-O prepared from 2 normal and 3 patient plasmas. Four curves were run on normals and 7 on v.W.d. plasmas and values averaged. Points

represent mean values and vertical lines a single standard error of mean. Fractions were heated without prior $Al(OH)_3$ adsorption and pH of each was maintained at 6.5 with citrate buffer(14).

FIG. 4. Effect of a variety of pH's was determined on AHF activity of 2 normal and 3 transfused v.W.d. plasmas. Each sample was run 3 times independently. After having been thawed and adsorbed with $Al(OH)_3$, pH of each plasma was brought to the desired point with 0.5 N HCl or NaOH. After having been maintained at designated pH for 30 min at 37°C, each sample was returned to pH 7.2 with the appropriate acid or base. Final concentrations were adjusted with saline and samples were centrifuged at 4°C and 3,000 rpm to remove precipitated proteins, then stored at 0°C until assayed for AHF activity.

FIG. 5. Thromboplastin generation tests (TGT) performed on $Al(OH)_3$ adsorbed plasmas. Topmost curve is one performed on v.W.d. #3 before transfusion. Other curves were produced by using a single normal and 4 transfused v.W.d. plasmas.

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Received December 16, 1963. P.S.E.B.M., 1964, v115.

Replacement Therapy with Calciferol in Thyroparathyroidectomized Lactating Rats.* (29031)

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Successful replacement therapy was achieved in thyroparathyroidectomized (TPEX) lactating rats using 60, 80 and 100 μg Hytakerol (A.T.10)/100 g body weight (BW)/day and 3 μg L-thyroxine (T_4)/100 g BW/day administered from day 7 to 13 of lactation(1). The effectivenes of A.T.10 in increasing milk secretion in normal rats was reported also(2).

It was claimed that Hytakerol contained 1.25 mg crystalline dihydrotachysterol (DHT) per ml whereas the new product is labeled to contain only 0.25 mg DHT per ml. It was reported, however, that Hytakerol does not contain DHT but is dihydrovitamin-D2 II, a chemical compound that can be obtained either by reduction of tachysterol or Vit. D2(3).

It is desirable, therefore, to test the effectiveness of the parent compound (Vit. D2 or calciferol) on lactation intensity in experimental animals. When 0.2 mg calciferol/100 g BW was given to normal lactating rats, an increase in milk yield of 39% above control on day 14 of lactation was observed (unpub.).

Materials and methods. Lactating rats of the Sprague-Dawley-Rolfsmeyer strain were

* Contribution from Mo. Agr. Exp. Sta., Journal Series No. 2670. Approved by Director.

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