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Research Article

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Antihemophilic Factor Antigen

LOCALIZATION IN ENDOTHELIAL CELLS BY IMMUNOFLUORESCENT MICROSCOPY

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ABSTRACT The tissue localization of antihemophilic factor (AHF, Factor VIII) has been determined by immunofluorescent studies using monospecific rabbit antibody to human AHF. Specific staining demonstrating AHF antigens has been identified in endothelial cells of a wide range of human tissues. The staining pattern was observed in endothelial cells of arteries, capillaries, and veins as well as the cells lining hepatic and splenic sinusoids. Specific fluorescence was limited to these endothelial cells in sections of kidney, liver, spleen, lymph node, cardiac and smooth muscle, thyroid, umbilical cord, and skin. Absorption studies established that the staining was specific for cells in which there were proteins that had AHF antigens. The demonstration of fluorescence within the cytoplasm of endothelial cells suggests that these cells synthesize proteins that have AHF antigens.

INTRODUCTION

Antihemophilic factor (AHF, Factor VIII)¹ procoagulant activity is reduced in patients with classic hemophilia (hemophilia A) and von Willebrand's disease. Despite considerable effort by many investigators in recent years, the cell type and/or organ responsible for the production of this coagulation factor has not been clearly established. Studies suggesting synthesis by liver

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¹ Abbreviations used in this paper: AHF, antihemophilic factor (Factor VIII); PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; VWF, von Willebrand's factor.

(1-5), spleen (2, 3, 6, 7), kidneys (8), lymphocytes (9), and macrophages (10) have been reported. These previous investigations have reported AHF synthesis and/or release from perfused organs (2, 3, 7), specific cell types in tissue culture (9, 10), and in AHF-deficient animals given organ transplants (4, 5). Such studies are limited by the lability of AHF procoagulant activity, however, and by the difficulty in distinguishing changes in AHF activity from nonspecific shortening of clotting-time assays by other procoagulant materials. As a result, numerous conflicting claims have not yet been resolved.

The use of monospecific antibodies to antihemophilic factor provides an alternative way to study AHF. It has been recently demonstrated that antisera from rabbits immunized with highly purified AHF inactivate AHF procoagulant activity (11-13), form stable complexes with AHF (13), and react with AHF in immunoprecipitation assays (11, 13). These properties suggest that anti-AHF might also be an effective reagent for morphologic study of AHF distribution. Our studies (14), and the concurrent independent investigations of Bloom, Giddings and Wilks (15), have verified this suggestion and have identified AHF within endothelial cells in human tissues using indirect immunofluorescent microscopy. The present report extends these findings to additional tissues, provides more detailed evidence for the specificity of this staining, and identifies with greater precision the localization of the immunoreactive material.

METHODS

Preparation of antisera. Rabbit anti-AHF was obtained from New Zealand albino rabbits immunized with highly purified AHF (80-100 U/mg protein) prepared from fresh normal human plasma by precipitation at -3°C with 3% ethanol, precipitation with 10% polyethylene glycol, and gel filtration through Sepharose 4B (Pharmacia Fine Chemi-

icals, Inc., Piscataway, N. J.) following the method of Zimmerman, Ratnoff, and Powell (11). The antiserum used in the studies reported here was obtained after a total of four injections over a 7 month period, and it was absorbed with an AHF-poor fraction of normal human plasma (13). A single line was identified when this absorbed serum was then tested by Ouchterlony gel diffusion against AHF-rich concentrates (3% ethanol at -3°C with suspension of the precipitate in $\frac{1}{10}$ th of the original volume) of normal and hemophilic plasmas, but no lines were observed when this absorbed antiserum was tested with plasma concentrates from patients with severe von Willebrand's disease. Immunoelectrophoresis of concentrates from normal and hemophilic plasmas demonstrated a single line with β -mobility; these findings are identical to those reported by Zimmerman and co-workers (11). This monospecific absorbed antiserum had the same capacity to inactivate AHF activity in normal human plasma as did the rabbit serum from which it was prepared. Further details of antigen preparation, immunization schedule, and absorption procedure have been reported (13).

This monospecific antiserum or a globulin fraction prepared from the serum by precipitation with 50% saturated ammonium sulfate followed by dialysis against 0.01 M phosphate, 0.14 M NaCl, pH 7.5 (PBS) was diluted in PBS before immunofluorescent staining using the indirect technique. Entirely consistent results were obtained whether serum (diluted 1:16) or globulin (0.2–0.8 mg/ml) was used.

Immunofluorescent methods. The studies were carried out in two separate laboratories using immunofluorescent methods (16, 17) that are identical except as noted below. 5-mm blocks of tissue obtained by surgical biopsy or at autopsy within 6 h of death were rapidly frozen on a small cellulose sponge in isopentane prechilled in liquid nitrogen or in a dry ice-ethanol bath and stored at -70°C until used (less than 3 mo). Sections were cut in a cryostat, air dried, and stored at -20°C for periods as long as 3 mo. Smears prepared from peripheral blood, aspirates of bone marrow, and platelet-rich plasma were air dried, fixed in acetone for 10 min, and stored at -20°C for 1 wk or less.

Fluorescent staining of tissue and smears was performed according to the method of Ortega and Mellors (18) using the indirect technique. Sections were air dried, washed three times with PBS (5 min/wash), and reacted with unlabeled antiserum for 30 min. They were then washed three times with PBS (2 min/wash) and treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit gamma globulin for 45 min. The sections were again washed three times with PBS (2 min/wash) and then covered with a drop of 10% glycerin, 90% PBS, and a glass cover slip. Longer periods of washing with PBS before and after exposure to the same unlabeled antiserum in one laboratory (16) did not affect the pattern of fluorescence. All incubations and washes were carried out at room temperature.

The goat anti-rabbit globulin was prepared in goats immunized with rabbit IgG that had been isolated by DEAE-cellulose chromatography. An IgG fraction of this goat antiserum, also obtained by DEAE-cellulose chromatography, was labeled with FITC (19) and rechromatographed using DEAE-cellulose (20). A single line was identified when the antiserum was tested with rabbit serum by immunoelectrophoresis, and it did not react with human serum in Ouchterlony gel diffusion. The F/P ratio (milli-

grams FITC/milligram protein) of the FITC-goat anti-rabbit globulin used in these studies was 7.3×10^{-3} . The FITC-goat anti-rabbit globulin was absorbed with human IgG (3 mg/ml antiserum) before use at a final concentration of 0.5–0.9 mg/ml. Similar immunofluorescence observations were made using FITC-goat anti-rabbit globulin obtained from Hyland Div. (Travenol Laboratories, Inc., Costa Mesa, Calif.) that was absorbed with lyophilized mouse liver powder before use at a 1:16 dilution.

Stained sections were examined in a Leitz (E. Leitz, Inc., Rockleigh, N. J.) Ortholux photomicroscope using an HBO 200 W light source, a UG1 (2 mm) excitation filter and Leitz UV absorbing barrier filter or with a Zeiss (Carl Zeiss, Inc., New York) microscope using a similar light source and a BG 12 excitation filter. In some instance, an FITC interference filter was used. Photographs were taken using Tri-X Pan film (Eastman Kodak Co., Rochester, N. Y.). Selected tissue sections were examined by phase contrast immunofluorescent microscopy as well using a Zeiss Ultraphot II microscope with indirect immunofluorescent illumination. This permitted more precise definition of the distribution of positive immunofluorescence. For the further identification of tissue, marrow, or blood cell elements, cover slips were removed after photographs of immunofluorescent staining were obtained. Sections or smears were then restained using hematoxylin-eosin for tissue and Wright's stain for bone marrow and blood smears.

Specificity of antiserum. The specificity of staining for AHF was evaluated by absorption studies. Anti-AHF globulin (0.05 ml of a globulin preparation that had a protein concentration of 13.2 mg/ml) was incubated with 0.25–0.5 ml of the absorbing material at 37°C for 1 h and at 4°C overnight. After the precipitate was removed by centrifugation (2,400 *g* for 30 min), the absorbed anti-AHF was used for immunofluorescent studies at the same dilution as anti-AHF to which PBS had been added instead of the absorbing material.

Ethanol concentrates were prepared from 5 ml of plasma by slowly adding 80% ethanol to a final concentration of 3% and holding the material at -3°C for 30 min before centrifuging (17,000 *g* for 20 min at -3°C). The material used in absorption studies was dissolved in 0.15 M NaCl to a final volume of 0.25 ml. A concentrate of normal human serum was obtained by precipitating proteins with one-third saturated ammonium sulfate and dialyzing the redissolved material against 0.15 M NaCl. The globulin obtained from 5 ml of serum was concentrated to 0.3 ml in this manner. Absorptions of the anti-AHF were, therefore, carried out with concentrates prepared from 100 times the volume of the antibody globulin preparation. Purified human fibrinogen prepared by the method of Takeda (21) contained greater than 95% coagulable protein and was used at a ratio of 29 mg/ml antibody globulin. Highly purified human AHF obtained by the method of van Mourik and Mochtar (22) was used at a ratio of 1 mg/ml antibody globulin. A comparison of the AHF procoagulant activity (1,400 U/100 ml) with protein content (0.10 mg/ml) indicates that the AHF preparation was approximately 10,000-fold purified when compared with normal plasma.

Control studies also included tissues incubated with rabbit antifibrinogen. Monospecific rabbit anti-human fibrinogen (Hyland Div., Travenol Laboratories) used for indirect fluorescent staining gave results consistent with those obtained when monospecific FITC-conjugated rabbit anti-human fibrinogen, prepared as previously described (17), was used for direct staining. Both antifibrinogen sera

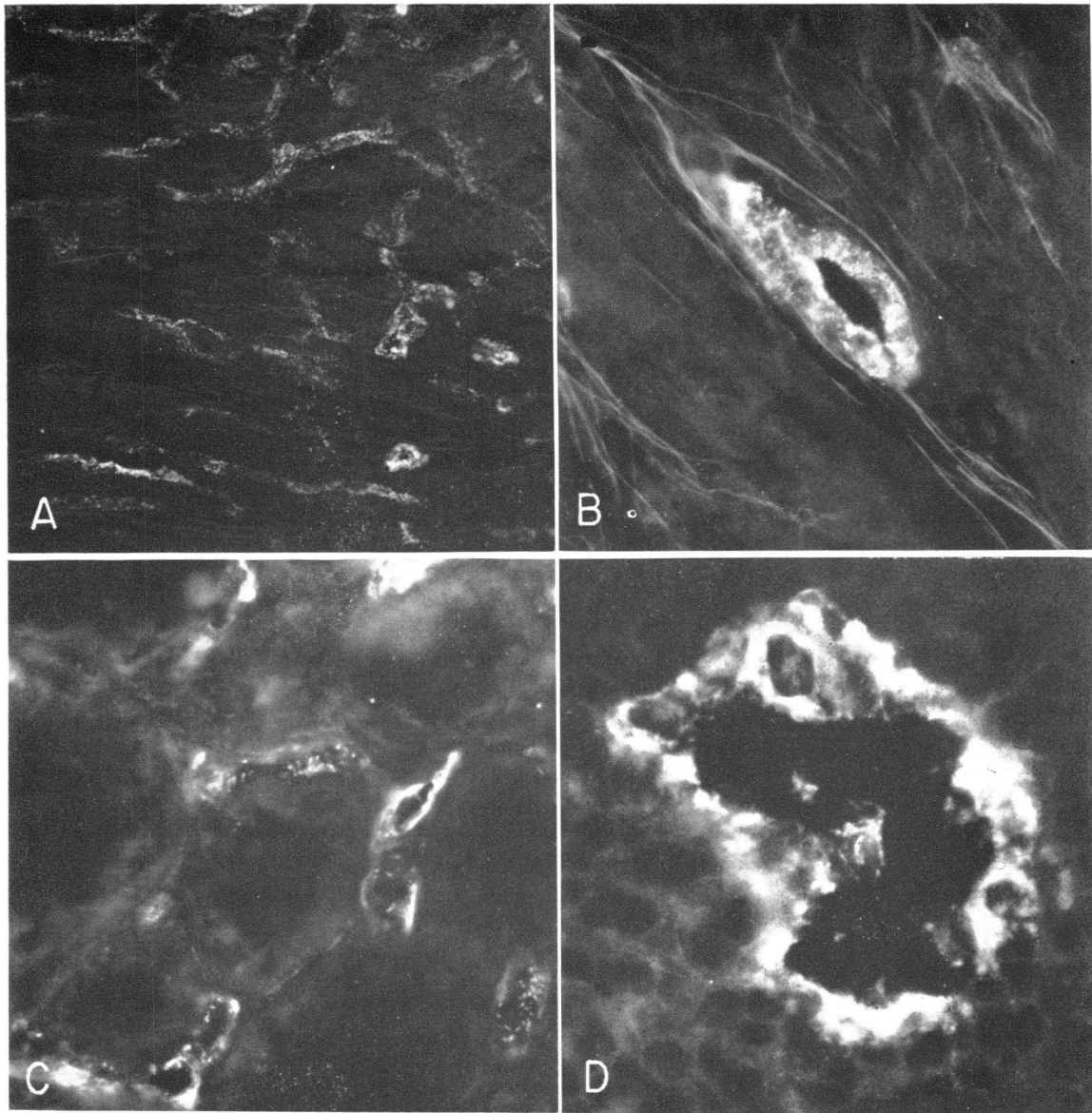


FIGURE 1 Immunofluorescent micrographs of tissue sections stained with rabbit anti-AHF. (A) Low power micrograph of cardiac muscle with positive staining of venules and capillaries cut longitudinally and in cross section ($\times 150$). (B) Higher power micrograph of the same cardiac muscle section. The positive staining is limited to endothelial cells of the small venule ($\times 350$). (C) Renal cortex with positive staining of peritubular capillaries ($\times 500$). (D) Lymph node venule showing positive staining of endothelial cell cytoplasm. The surrounding lymphoid cells are negative ($\times 1,200$).

formed single immunoprecipitin lines when tested with human plasma by Ouchterlony immunodiffusion analysis and by immunoelectrophoresis.

RESULTS

Sections of liver, spleen, lymph node, kidney, skeletal and cardiac muscle, thyroid, skin, umbilical cord, aorta, and portal vein were examined using indirect immunofluorescent microscopy. Specimens that were obtained

by surgical biopsy from individuals with no history of bleeding or thrombotic disorders included skin, kidney, muscle, and spleen. These included 20 kidney specimens obtained from living, related kidney-transplant donors immediately after removal of the kidney for transplantation. These individuals gave specific informed consent for research studies utilizing the biopsy material. Additional material was obtained at autopsy of patients with

no history of bleeding or thrombotic disorders. Sections from five livers, four spleens, four lymph nodes, two umbilical cords, and two skin biopsies were examined. One sample was examined for each of the other tissues listed above.

In all tissues examined, immunofluorescent staining demonstrating AHF antigenic material was restricted to endothelial cells (Figs. 1 and 2). This endothelial localization was apparent in arteries, capillaries, and venules and was observed in sections of aorta and portal vein. The distribution of positive immunofluorescence within endothelial cells was cytoplasmic and in most cases appeared to be granular. The nuclei of these cells did not stain and were seen as negative images (Fig. 1D). In those tissues in which the endothelial cytoplasm is very thin (e.g., arteries and some glomerular capillaries), the immunofluorescent staining appeared more linear (Fig. 2A). The density of endothelial cells within the red pulp of the spleen made it difficult to be absolutely certain that each positively staining cell was endothelial. Phase-contrast immunofluorescence improved the definition of the distribution, however, and at least 95% of positively staining cells in the spleen were identified as endothelial cells. Staining was not identified within vascular lumens.

Lymphocytes and plasma cells in lymph node and spleen sections did not show positive staining for AHF

(Fig. 1D). Similar findings were observed in smears prepared from peripheral blood and from aspirated bone marrow. Neither normal marrow lymphocytes nor peripheral blood lymphoblasts from a patient with acute lymphoblastic leukemia were stained by the rabbit anti-AHF. Megakaryocytes were the only marrow cells showing positive staining for AHF. Similarly, only platelets were stained when peripheral blood smears were examined. Macrophages identified in marrow smears were not stained.

The immunofluorescent staining of renal tissue sections was restricted to endothelial cells and was prominent in arteries (Fig. 2A) and arterioles, venules, and capillaries (Fig. 1C). Within glomeruli the staining, restricted to endothelial cells, was linear or granular within the cytoplasm according to the plane on which the section was cut. Sections of liver had prominent fluorescent staining of veins in portal triads as well as sinusoidal lining cells, but parenchymal cells were not stained by rabbit anti-AHF. Control experiments with rabbit anti-fibrinogen demonstrated fluorescence restricted to liver parenchymal cells and there was no staining of endothelial cells.

The pattern of immunofluorescence was generally consistent from tissue to tissue and within sections. Although there was some variability in the intensity of staining for AHF, positive endothelial staining was ob-

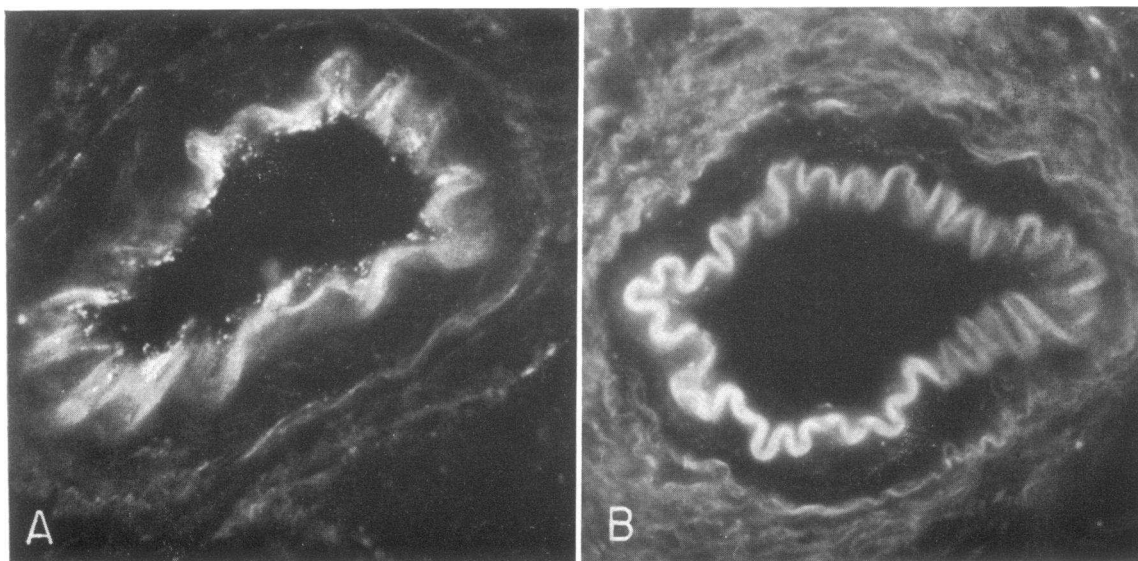


FIGURE 2 Cross section of small muscular arteries in the renal cortex. (A) Section stained with rabbit anti-AHF demonstrating positive staining of endothelial cells lying on the luminal side of the internal elastic lamina. The autofluorescence of the internal elastic lamina should be distinguished from the granular endothelial cell staining. The autofluorescence is present in stained tissues by its blue color which is easily distinguished from the apple green color of fluorescein-stained material ($\times 800$). (B) Section stained with rabbit antihuman fibrinogen. There is no positive staining of endothelial cells. Prominent autofluorescence of the internal elastic lamina is also present in this artery which is from a different field from the same tissue section ($\times 800$).

served in all tissue sections examined. Specimens obtained by surgical biopsy had the same immunofluorescent patterns as those in tissue obtained at autopsy. Although there were minor differences in immunofluorescent methods, the staining patterns observed in the two laboratories did not differ.

The specificity of the immunofluorescent staining was established in a series of control studies. After absorption of the anti-AHF with 0.075 mg of highly purified AHF/mg IgG, the antiserum no longer stained tissues that strongly reacted with unabsorbed anti-AHF (Table I). Positive staining of endothelial cells was also completely blocked by absorption of rabbit anti-AHF with concentrates prepared from normal human plasma, normal serum, and from plasma of a patient with hemophilia A. In contrast, absorption with an ethanol concentrate of plasma from a patient with severe von Willebrand's disease (AHF activity less than 2 U/100 ml [13]) did not decrease positive staining of endothelial cells. Absorption with purified human fibrinogen (2.2 mg/mg antibody IgG) also failed to affect endothelial cell staining.

Tissue sections incubated with normal rabbit serum, normal rabbit IgG, rabbit antihuman fibrinogen, or PBS followed by FITC-goat antirabbit globulin showed no positive staining of endothelial cells. Hepatic parenchymal cells were stained when rabbit antifibrinogen was used, and absorption of this antibody with highly purified AHF failed to decrease the staining of parenchymal cells for fibrinogen. The absorption studies are summarized in Table I.

DISCUSSION

The immunofluorescent microscopic studies reported here demonstrate the localization of AHF antigen in endothelial cells throughout the body. The specific staining was detected in the endothelial lining of arterial and venous vessels as well as in capillaries and cells lining splenic and hepatic sinusoids. No immunofluorescent staining was identified in lymphocytes or macrophages—cells previously considered possible sources of AHF—or in hepatic or renal parenchymal cells. The similar distribution in biopsy and autopsy material is evidence that the localization is not related to diffusion of AHF into—or adsorption onto—nonviable endothelial cells. The staining pattern, cytoplasmic rather than surface, is additional support for this conclusion.

Control studies (Table I) established that the staining was specific and that the rabbit anti-AHF has the same properties in immunofluorescence microscopy as it does in immunoprecipitation (11), antibody neutralization (11), and radioimmunoassays (13). The antibody reacts with—or is absorbed by—normal and hemophilic plasma and serum and by highly purified AHF. It does not react with plasma from patients with severe von Willebrand's

TABLE I
Effect of Antibody Absorption on Endothelial Cell Fluorescence

Antiserum	Absorbing antigen*	Endothelial cell immunofluorescence‡
Anti-AHF	—	+
Anti-AHF	Purified AHF	—
Anti-AHF	Fibrinogen	+
Anti-AHF	Normal plasma	—
Anti-AHF	Hemophilic plasma	—
Anti-AHF	von Willebrand's disease plasma	+
Anti-AHF	Normal serum	—
Normal rabbit serum	—	—
Antifibrinogen	—	—

* The properties of the absorbing antigens and the ratios of antigen to anti-AHF are specified in the text.

‡ All sections were incubated with FITC-goat antirabbit globulin after the absorbed rabbit antiserum.

disease or with plasma proteins other than AHF, e.g., fibrinogen.

Although the control studies indicate that the rabbit antibody specifically stains endothelial cells, the conclusion that this represents the identification of AHF antigen in these cells requires evidence that the rabbit antibody does, in fact, specifically react with the same molecule in normal human plasma as that which is responsible for AHF procoagulant activity.

Four kinds of evidence support this conclusion. The most direct is the demonstration that this antiserum forms a single line in immunodiffusion studies with highly purified AHF and with AHF-rich concentrates of normal human plasma (11, 13). By immunochemical criteria, this antiserum has a single specificity. No precipitin line is detected, however, if the rabbit antiserum is tested with concentrates prepared from plasmas of patients with severe von Willebrand's disease (11). The parallel reduction of AHF procoagulant activity and AHF antigenic material in von Willebrand's disease plasmas is additional evidence that this antibody reacts with the AHF molecule (11, 13). The similar physicochemical properties of AHF procoagulant activity and the antigen identified by rabbit anti-AHF provide a third kind of evidence for the specificity of the rabbit antibody (13).

Unambiguous evidence for the antibody's specificity has recently been obtained by immunizing rabbits with washed immunoprecipitates formed by incubating concentrates of normal human plasma with the rabbit anti-AHF (23). Postimmunization sera from these animals had antibodies that inactivate AHF procoagulant activity.

This observation demonstrates the presence of proteins with AHF determinants in the immunoprecipitates. The specificities identified by anti-AHF are, therefore, designated "AHF antigens," and the proteins with which they react (if nonfunctional) are recognized as being "AHF-like."

It is possible, of course, that the more sensitive immunofluorescent method might identify immunoreactive material that cannot be detected in immunoprecipitation studies. The absorption studies using concentrates of normal and von Willebrand's plasmas strongly suggest, however, that the immunofluorescent pattern reflects tissue localization of AHF antigens.

The molecule identified by rabbit anti-AHF appears to have a second hemostatic role, that of "von Willebrand's factor" (VWF), in addition to its AHF procoagulant function. Defective platelet function—and a prolonged bleeding time—in von Willebrand's disease is attributed to VWF deficiency and fractions of normal plasma correct these abnormalities *in vivo* and *in vitro* (24–28). As AHF procoagulant activity and VWF are usually present in the same fractions obtained from normal plasma, it has been suspected for many years that the two activities might be present on a single molecule. Bouma, Wiegerinck, Sixma, van Mourik, and Mochtar have recently obtained additional immunologic evidence that closely links AHF and VWF (29). They demonstrated that high molecular weight fractions prepared by agarose gel filtration of normal or hemophilic plasma correct the reduced platelet retention (in glass-bead filters) of blood from patients with von Willebrand's disease and that these are the same fractions that have the highest concentrations of AHF procoagulant activity and AHF antigen. Moreover, rabbit anti-AHF reduces platelet retention of normal blood (29).

Weiss, Rogers, and Brand, using an *in vitro* assay for VWF based on ristocetin-induced platelet aggregation, have also demonstrated VWF in high molecular weight fractions obtained by agarose gel filtration of cryoprecipitates prepared from normal and hemophilic plasmas (30). Rabbit anti-AHF also inhibits VWF activity measured in this way (30). It appears likely, therefore, that the hemostatic defects in von Willebrand's disease follow from the reduced concentration of a single molecule (or a relatively stable complex of molecules) that has both AHF and VWF activity. It is recognized that the close correlation of VWF and the antigenic determinants detected by rabbit anti-AHF has important implications for the studies reported here. The tissue localization of both VWF and AHF are identified by the endothelial cell immunofluorescence.

While immunofluorescent studies cannot establish that the intact and functional AHF or VWF are present in—or synthesized by—the endothelial cells, they do provide

a guide for more probing studies. Human endothelial cells in tissue culture provide a more direct way to study the relationship of AHF and VWF to this cell type (31). The accompanying paper reports studies that demonstrate positive immunofluorescence in cultured human endothelial cells (but not other tissue culture cells) when they are tested with rabbit anti-AHF (32). In addition, proteins that have AHF antigenic determinants are synthesized by these endothelial cells (32). In conjunction with these tissue culture studies, the immunofluorescent histologic observations strongly suggest that the endothelial cell synthesizes proteins that have AHF antigens.

Bloom, Giddings, and Wilks have suggested an alternative interpretation, the adsorption of AHF from plasma by endothelial cells, for their similar indirect immunofluorescent results (15). A thin but well-demarcated layer of immunoreactive material lining the endothelium of large and small arteries and veins was identified using rabbit anti-AHF (15). Our findings were similar when sections were observed at low magnifications, but it is apparent in higher resolution photomicrographs that fluorescent staining is detected throughout the cytoplasm of endothelial cells (Figs. 1B and 1D). This cytoplasmic distribution would appear more consistent with AHF antigen synthesis by endothelial cells, an interpretation supported by tissue culture data (32).

Bloom and co-workers also detected, but did not illustrate, immunofluorescence in cytoplasm of mononuclear cells of the spleen and lymph nodes and perceived faint and uncertain staining of hepatic parenchymal cells. Lymphoid and hepatic parenchymal cells showed no positive staining for AHF in our studies. Although the rabbit antisera to AHF were prepared by generally similar methods, differences in antigen preparation and absorption may account for differences in the localization of immunofluorescence reported.

An earlier immunofluorescent study of AHF within tissues reported localization in bovine splenic lymphocytes and hepatic parenchymal cells (33). That report did not include criteria establishing the specificity of the antibody, however, and it is possible that the findings were related to other antigens in the immunizing material.

The localization of AHF antigen in the endothelial cell is consistent with a number of tissue extraction and organ perfusion studies in which AHF has been identified by assays of procoagulant activity (2, 3, 6–8) and an immunologic study in which antibody neutralization was measured (34). Vascular organs—liver, kidney, and spleen—have been reported to be the source of AHF in these studies, as would be expected if the appearance of AHF in extracts or perfusates is related to the synthesis and release of AHF by endothelial cells. Although tissue culture studies of lymphocytes and fibroblasts have

been interpreted as indicating AHF synthesis by these cells, immunologic determinations have established that the procoagulant material in cultured cells is not AHF and that the shortened coagulation times are due to tissue factor (35-37).

The endothelial cell localization of AHF antigen is of considerable interest in light of the marked lability of plasma AHF procoagulant activity values. Rapid increase is noted after exercise, injection of epinephrine, neurologic procedures, surgery, and a range of other stressful stimuli (38). Parallel increases in AHF antigen values have been identified in many of these conditions (39-40). The mechanism by which these stimuli cause increased plasma AHF levels is not known.

It may be significant that tissue thromboplastin has also been identified in endothelial cells (41). The vascular localization of these procoagulant materials, and possibly others as well, would expedite their rapid interaction in the hemostatic sequence and may, therefore, have considerable survival value. It is also possible that their endothelial localization is an important factor in the development of thrombosis. If minimal changes in the endothelial cells effect significant alterations in the blood coagulation system, these cells would have a central role in the pathogenesis of thrombotic diseases. Further studies of the procoagulant functions of endothelial cells are needed to consider this possibility.

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