Localization of Factor VIII-Procoagulant Antigen: An Immunohistological Survey of the Human Body Using Monoclonal Antibodies

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Various organs, including liver, spleen, heart, lung, kidney, intestines, lymph nodes, pancreas, bone marrow, and thymus, were investigated for the presence of factor VIII-procoagulant antigen (VIIICAg) and factor VIII-related antigen (VIIIIRAg). Using a panel of monoclonal antibodies directed to factor VIII-von Willebrand factor in combination with a sensitive immunoperoxidase staining technique. In addition to hepatic sinusoidal endothelial cells, the presence of VIIICAg was demonstrated in mononuclear cells sporadically present in lymph nodes, in the alveolar septum of lung, and in the red pulp of spleen. The identity of these mononuclear cells could not be unequivocally determined. Based on morphological criteria, however, it is tentatively concluded that these cells are nonlymphoid and belong to the mononuclear phagocyte system. The presence of VIIIIRAg was confined to vascular endothelial cells, hepatic sinusoidal endothelial cells, cells lining the venous sinuses of the red pulp of the spleen, cells lining renal glomeruli and lung capillaries, platelets, and megakaryocytes.

FACTORS VIII-von Willebrand factor (FVIII-vWF), a high-molecular-weight glycoprotein complex that has an important role in hemostasis, consists of two discernible components. These are factor VIII, the procoagulant component absent in severe hemophilia A, and von Willebrand factor (vWF), the component that is absent in severe von Willebrand's disease.1 Immunohistological2 and recombinant DNA studies3-5 have indicated that factor VIII is present in and synthesized by liver cells. From earlier transplantation and perfusion studies,6-8 circumstantial evidence was obtained that the liver is involved in the synthesis of factor VIII; however, a variety of other organs have been implicated in the synthesis of factor VIII as well.9-11 Hence the possibility of extrahepatic synthesis of factor VIII cannot be excluded in advance. This prompted us to extend the immunohistological search for VIIICAg to various other organs and cell types by use of a panel of monoclonal antibodies with different specificity to factor VIII. In addition, parallel experiments were performed using monoclonal antibodies against vWF.

It appeared that the presence of factor VIII was restricted to two cell types: hepatic sinusoidal endothelial cells and mononuclear cells, found sporadically but consistently in lung, spleen, and lymph nodes.

MATERIALS AND METHODS

Monoclonal antibodies. For this study eight monoclonal antibodies to factor VIII and four monoclonal antibodies to vWF were used. Description of techniques for characterization of these antibodies have been provided elsewhere and are applicable for this study.12 Some of these antibodies have already been described in detail elsewhere.13-16 In brief, monoclonal antibodies CLB-CAg A (IgG2a), CLB-CAg 21 (IgG2b), CLB-CAg 44 (IgG2b), CLB-CAg 58 (IgG2a), CLB-CAg 61 (IgG2b), CLB-CAg 78 (IgG2b), CLB-CAg 117 (IgG2a), and C 6 (IgG1, IgG3) are directed to factor VIII as judged by the following criteria: they bind to purified FVIII-vWF17 and to FVIII-vWF present in normal plasma, but they do not react with severe hemophilia A plasma (negative for material cross-reacting with a human antibody to VIIICAg). Some of these antibodies inhibit factor VIII-procoagulant activity (VIIIC): C6 (5,000 Bethesda units (BU)/mg IgG), CLB-CAg A (150 BU/mg IgG), CLB-CAg 58 (5 BU/mg IgG), and CLB-CAg 117 (2 BU/mg IgG), whereas the other monoclonal antibodies do not inhibit VIIIC. All these monoclonal antibodies react with serum VIIICAg, except antibody C6, which suggests that the C6 antibody is specific for the native conformation of the factor VIII molecule. As deduced from the results of competitive displacement studies carried out essentially as described by Stel et al,18 these antibodies were directed to different epitopes on factor VIII. Monoclonal antibodies CLB-RAg 1 (IgG1), CLB-RAg 7 (IgG1), CLB-RAg 35 (IgG1), and CLB-RAg 50 (IgG1) are directed to vWF15,16: they bind to purified FVIII-vWF, to FVIII-vWF present in normal plasma, and to FVIII-vWF present in severe hemophilia A plasma. In contrast, these antibodies do not react with plasmas of patients with severe von Willebrand's disease (negative for material cross-reacting with a rabbit antibody to vWF). Competitive displacement studies showed that these antibodies are directed to different epitopes on vWF.

Immunoperoxidase staining procedure. A sensitive multistep immunoperoxidase staining method was used to demonstrate the presence of VIIICAg. This procedure has been described in detail by Lansdorp et al.19 Sheep antimouse immunoglobulin (SAM) (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service [CLB], Amsterdam) was used to link peroxidase-mouse monoclonal antiperoxidase complexes (PAP) (CLB) to the initial monoclonal antibody (eg, monoclonal antibody to VIIICAg). The multistep PAP procedure was based on repeated subsequent incubation cycles with SAM and PAP complexes, respectively. The experimental details of the immunostaining procedure were as follows: (1) cryostat sections of 5-μ thickness were air-dried, fixed in acetone for ten minutes, and washed in phosphate-buffered saline (PBS) for ten minutes; (2) monoclonal antibodies (undiluted culture supernatant or ascitic fluid diluted 1:400 to 1:1,000 in PBS-gelatine 0.2%) were overlaid on the tissue and allowed to incubate for two hours at room temperature; (3) SAM, diluted 1:100 in 0.1 mol/L Tris-HCl (pH 7.5) containing 10% normal human serum and 10% normal sheep serum, was applied to the slides. Incubation was allowed for 30 minutes at room temperature; (4) PAP complexes, diluted 1:100 in the above-mentioned Tris-HCl buffer containing 10% normal sheep serum and 10% normal human serum, were overlaid on the tissue for 30 minutes. Between steps 2, 3, and 4, and 5, the sections were washed in PBS for five minutes. To improve the sensitivity of the immunoperoxidase staining procedure, steps 3 and 4 were repeated twice before (5) peroxidase activity was detected using the substrate 3,3'-diaminobenzidine (DAB) (Sigma Chemical Co, St Louis) (10006-4971/86/6701-0035$03.00/O)
mg DAB/mL in PBS containing 0.01% hydrogen peroxidase). After five to ten minutes' incubation in the dark, the sections were washed in PBS, dehydrated, and mounted with Malinol (Chroma, Stuttgart, FRG). Similar immunostaining results were obtained when, in steps 3 and 4 of the immunostaining procedure, normal serum was substituted by serum obtained from a patient with severe von Willebrand's disease. The possibility that all immunostaining results obtained with the monoclonal antibodies to FVIII-vWF were due to Fc-binding of these antibodies to the stained cells was excluded by the absence of reactivity with monoclonal antibodies directed to unrelated antigens of the same immunoglobulin subclasses and by the finding that saturation of Fc receptors by preincubation with aggregated gamma globulins had no effect on the immunostaining results obtained with the monoclonal antibodies to FVIII-vWF. The monoclonal antibodies to unrelated antigens used were a monoclonal antibody directed to T lymphocytes (IgG,) (Becton Dickinson, Antwerp, Belgium), to mouse thymocytes (IgG3) (kindly provided by Dr W.v. Ewijk, Erasmus University, Rotterdam, The Netherlands), to trinitrophenyl (IgG), to house dust mite (IgE), and to cat allergen (IgG3). The latter three monoclonal antibodies were kindly provided by Dr R.C. Aalberse, CLB, Amsterdam, The Netherlands.

Tissues. Tissues (pancreas, intestines, liver, kidney, lymph node, lung, heart, thymus, vena cava, and aorta) were collected at autopsies on patients deceased less than three hours. Tissue that showed no abnormality was selected for immunohistological study. Tissue of liver, kidney, and lymph node were also obtained through biopsy. Spleens were obtained from patients during surgery. Small tissue blocks were quick frozen and stored in liquid nitrogen. Bone marrow was aspirated by sternum puncture, and cytocentrifuge smears were made before immunoperoxidase staining.

Cultured endothelial cells. Endothelial cells isolated from human umbilical veins were cultured until confluent monolayers were obtained. After removal of culture medium and washing with PBS, the endothelial cells were incubated with RPMI 1640 (GIBCO, Biocult, UK) supplemented with 1% (wt/vol) human serum albumin. After 48 hours' incubation, samples were taken from the supernatant medium and stored at −70 °C until assayed for VIIICAg and VIIIRAg. For immunohistological studies, endothelial cells were cultured on glass coverslips until confluency was reached. After several washes with PBS to remove traces serum, cells were fixed in acetone for ten minutes and washed with PBS before immunoperoxidase staining.

Determination of VIIICAg and VIIIRAg. VIIICAg was measured using an immunoradiometric assay relying on Sepharose-linked monoclonal antibodies and human antibodies to VIIICAg. The lower limit of detection with this assay is 0.0005 units of VIIICAg per milliliter. Determination of VIIIRAg was essentially as described by Ruggeri et al. One unit of VIIICAg and VIIIRAg is the amount of antigen present in 1 mL of pooled normal plasma.

RESULTS

Localization of factor VIII (VIIICAg) in liver tissue. The localization of VIIICAg in liver biopsy material from 20 patients with mild or no detectable liver disease was investigated with an immunoperoxidase staining procedure using eight monoclonal antibodies directed against different epitopes on factor VIII. With each of these monoclonal antibodies, the most consistent finding was labeling of the cells lining the hepatic sinusoidal spaces (Figs 1 and 2). Diffuse as well as granular deposition of the immunoperoxidase reaction product could be observed in the cytoplasm of these cells (Fig 3). Hepatic parenchymal cells, as well as the vascular endothelial cells lining hepatic arteries and veins, were negative. No subendothelial deposition of immunoreactive material around arteries and veins was observed. Similarly, when liver tissue obtained from five patients with severe hepatic parenchymal disease (severe cirrhosis) was tested with these antibodies, it appeared that the cells lining the liver sinusoids were positive (results not shown). The observed immunostaining patterns obtained with these eight anti-VIIICAg monoclonal antibodies were similar, and only slight differences in staining intensity were occasionally present. Labeling of the sinusoidal liver cells could only be demonstrated by repeating the incubation cycles in the immunostaining procedure (see Materials and Methods). Apparently, on single incubation cycle in the immunostaining procedure was not sufficient to visualize the immunoreactive material present in these cells; therefore, in all subse-
Fig 3. Liver section stained with anti-VIIICAg monoclonal antibody CLB-CAG A. Endothelial cell (arrow) lining the sinusoid shows strong granular cytoplasmatic staining (counterstained with hematoxylin). (Original magnification ×1,000; current magnification ×790; multistep immunostaining procedure.) S, sinusoid; EC, nucleus of sinusoidal endothelial cell; H, nucleus of hepatocyte.

sequent experiments, we used the multistep immunostaining procedure.

In control experiments, monoclonal antibodies directed to unrelated antigens of the same subclasses as the monoclonal antibodies to VIIICAg tested were negative (Fig 4).

Localization of vWF (VIIIRAg) in liver tissue. When monoclonal antibodies to VIIIRAg were tested on liver biopsy material from 25 patients with absent, mild, or severe hepatic disease, deposition of the immunoperoxidase reaction product, both diffuse and granular, was present in the endothelial cells lining hepatic arteries and veins, as well as in the sinusoidal liver cells (Fig 5). Subendothelial deposition of immunoreactive material was noted around hepatic arteries and veins. The sinusoidal staining pattern obtained with these anti-VIIIRAg antibodies was similar to that obtained with the anti-VIIICAg antibodies (compare Figs 1 and 5). As in the case of VIIICAg, the presence of VIIIRAg in the sinusoidal liver cells could only be consistently demonstrated after repetition of the incubation cycles in the immunostaining procedure. In contrast, the presence of VIIIRAg in vascular endothelial cells was readily detectable after only one cycle of immunostaining. The most likely explanation is that this reflects differences in the amount of antigen present in these cells.

Identification of Kupffer cells in liver sinusoids. An antibody directed to lysozyme (Dako, Glostrup, Denmark) was used to identify the Kupffer cells present in the liver sinusoids. As shown in Fig 6, the Kupffer cells represent a minority of the sinusoidal liver cell population, most of these cells being sinusoidal endothelial cells.22,23

Fig 4. Liver section incubated with a monoclonal antibody directed against trinitrophenyl. No immunostaining is present (original magnification ×250; current magnification ×212; multistep immunostaining procedure). S, sinusoid.

Fig 5. Immunoperoxidase staining with anti-VIIIRAg monoclonal antibody CLB-RAG 35. Section of liver tissue showing staining of sinusoidal endothelial cells (white arrow) and vascular endothelial cells (black arrow). (Original magnification ×125; current magnification ×81; multistep immunostaining procedure.)

Fig 6. Immunoperoxidase staining with an antibody against lysozyme. Section of liver showing staining of Kupffer cells (K) lining the hepatic sinusoids. Counterstained with hematoxylin (original magnification ×250; current magnification ×162; one-step immunostaining procedure). h, nucleus of hepatocyte; S, sinusoid.
Localization of factor VIII and vWF in other organs. Vascular endothelial cells present in other organs (lung, spleen, lymph nodes, pancreas, intestines, heart, thymus) and the endothelial cells lining the larger blood vessels (aorta, vena cava, endocard) did not stain with any of the monoclonal antibodies to VIIICAg tested. Occasionally, a faint, scattered staining in the glomerulus of the kidney was observed; however, we were not able to confirm this unequivocally despite the recent detection of factor VIII-mRNA in renal tissue. No VIIICAg could be detected in the sinusoidal endothelial cells lining the venous sinuses in the red pulp of the spleen; however, immunostaining was noticed in mononuclear cells sporadically present in the red pulp of the spleen (Fig 7), the alveolar septa of the lungs (not shown), and in lymph nodes (Fig 8). These cells, consistently found in these tissues, were stained by all eight monoclonal antibodies to VIIICAg. It appeared that the immunostaining was weak and diffuse, in contrast with the observed immunostaining in the cells lining the liver sinusoids. As deduced from the relatively large cell size and small nucleus/cytoplasm ratio, these mononuclear cells are presumably nonlymphoid and part of the mononuclear phagocyte system (probably macrophages).

Positive immunostaining for VIIIRAg was present in the cytoplasm of endothelial cells lining large and small arteries, veins, and capillaries. The endothelial cells lining the renal glomeruli and lung capillaries and the cells lining the venous sinuses in the spleen were also positive (Fig 9). Staining was both diffuse and granular, and this specific staining was achieved with all four of the tested monoclonal antibodies to VIIIRAg. Subendothelial deposition of VIIIRAg could only be discerned in arteries, arterioles, and larger veins. The multistep immunostaining procedure was essential to obtain consistent positive results for the cells lining the venous sinuses in the spleen and for the glomerular endothelial cells present in the kidney. By contrast, neither in the cells lining the lymphatic vessels nor in the cells lining the lymph node sinuses could VIIIRAg be demonstrated. In addition, repeated testing with the anti-VIIIRAg antibodies of lung, lymph node, and spleen tissue failed to reveal staining for VIIIRAg in mononuclear cells present in these tissues.

Bone marrow. None of the monoclonal antibodies to VIIICAg reacted with cells present in bone marrow. Megakaryocytes and platelets, however, showed immunostaining with all monoclonal antibodies to VIIIRAg that were tested (results not shown).

Cultured endothelial cells. The immunostaining experiments were repeated on cultured endothelial cells derived from human umbilical veins. In contrast with the monoclonal antibodies to VIIIRAg, which showed a diffuse and granular staining, the monoclonal antibodies to VIIICAg did not react with these cells. In addition, with immunoradiometric assays capable of detecting an amount of (serum) VIIICAg as low as 0.0005 U/mL, no VIIICAg could be demonstrated in the culture supernatant, although amounts of

![Fig 7](image-url). Section of spleen (red pulp) stained with anti-VIIICAg monoclonal antibody CLB-CAg 58. Note the labeling of mononuclear cells (arrow) (original magnification ×125; current magnification ×81; multistep immunostaining procedure). A, spleen sinus; G, granulocyte.

![Fig 8](image-url). Section of lymph node (paracortical area) stained with anti-VIIICAg monoclonal antibody C6. Note the labeling of mononuclear cells (arrow). (Original magnification ×125; current magnification ×112; multistep immunostaining procedure.)

![Fig 9](image-url). Section of spleen (red pulp) stained with anti-VIIIRAg monoclonal antibody CLB-RAg 1. The cells lining the red pulp sinuses (S) and the vascular endothelial cells show deposition of the peroxidase reaction product (original magnification ×50; current magnification ×32; multistep immunostaining procedure). V, splenic vein; A, splenic arteriole; G, granulocyte.
VIIIIRAg were readily detectable (25 mU VIIIIRAg/10^5 endothelial cells per 48 hours).

**DISCUSSION**

Using a sensitive immunostaining technique and monoclonal antibodies with different specificity against FVIII-vWF, several organs from the human body have been screened for the presence of factor VIII and vWF. The presence of material immunoreactive with anti-VIIIICAg monoclonal antibodies, and hence representing VIIIICAg, could be demonstrated in at least two cell types: (1) in cells lining the hepatic sinusoidal spaces, thus confirming results reported previously with only one monoclonal antibody to VIIIICAg, and (2) in mononuclear nonlymphoid cells occurring sporadically in lung, spleen, and lymph nodes. The latter results are corroborated by the work of Exner et al., who reported a relatively high concentration of VIIIICAg in lymph nodes and lung. Of those cells that were positive for factor VIII, the liver sinusoidal cells contained both VIIIICAg and VIIIIRAg, whereas in the mononuclear cells, only VIIIICAg seemed to be present. The identity of the latter cells could not be unequivocally determined. Judging by morphological criteria (size of cell and nucleus), we tentatively propose a subset of macrophages as the most likely candidate. With regard to the identity of the VIIIICAg containing liver sinusoidal cells, both Kupffer cells and sinusoidal cells can be considered as candidates. In view of the differences in staining patterns obtained with anti-VIIIICAg antibodies (Fig 2) and with antiisozyme antibodies (Fig 6) (the latter being a marker for Kupffer cells), it seems likely that the presence of VIIIICAg in liver is not restricted to Kupffer cells. The staining pattern obtained with the anti-VIIIICAg and anti-VIIIIRAg antibodies is more characteristic for hepatic sinusoidal endothelial cells, which outnumber the more patchily distributed Kupffer cells in the liver sinusoids. Although the involvement of Kupffer cells cannot be excluded, the hepatic sinusoidal endothelial cells are the most likely candidates to contain both VIIIICAg and VIIIIRAg. In the case of VIIIIRAg, this view is supported by data reported by Soda and Tavassoli. Using an indirect immunofluorescence technique in combination with a polyclonal rabbit anti-vWF serum, they showed that in liver suspension of rat Kupffer cells and sinusoidal endothelial cells, only the sinusoidal endothelial cells contained VIIIIRAg.

Based on the combined results of the present immunohistological study and previous recombinant DNA studies, we favor the concept that hepatic sinusoidal endothelial cells provide a site of synthesis for factor VIII.

The possibility that factor VIII is synthesized by hepatic parenchymal cells and, subsequently, stored in sinusoidal endothelial cells seems unlikely to us, since patients in the terminal stage of hepatic parenchymal disease have normal or elevated levels of factor VIII and reduced levels of vitamin K-dependent clotting factors. The latter known to be synthesized by liver parenchymal cells. The results of the present study, which show no deviate sinusoidal immunostaining with anti-VIIIICAg antibodies in patients with severe hepatic parenchymal disease, are consistent with these findings.

The significance of the presence of factor VIII in mononuclear cells (probably macrophages) occurring in spleen, lung, and lymph nodes is unclear. Macrophages, with their strong phagocytic and degrading capacities, are potential sites of clearance of factor VIII. On the other hand, macrophages have been frequently reported to actively synthesize a remarkable variety of substances, including clotting factors. In this respect, it is interesting to note that these mononuclear cells reacted with anti-factor VIII monoclonal antibody C6, which does not react with serum (degraded) VIIIICAg. Although this indicates that at least part of the factor VIII molecules are present in their native form, the possibility of uptake of native factor VIII cannot be ruled out. It will be clear that the issue synthesis or absorption cannot be resolved with immunohistological studies alone.

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