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# ASYMPTOMATIC STRUCTURAL LIVER DISEASE IN HEMOPHILIA

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**Abstract** In a study of persistent abnormalities of liver-function tests in hemophilic patients deficient in factor VIII or IX and treated with factor VIII or IX concentrates, we examined 14 liver biopsies from 13 anti-HBs-positive patients. None had any symptoms of liver disease. All had chronically abnormal levels of alanine aminotransferase. Histologic studies showed chronic persistent hepatitis in eight patients, chronic active hepatitis in four and fatty infiltration with portal fibrosis in one. Indirect immunofluorescence of antiserums containing anti-HBs or anti-HBc (or both) revealed nuclear and cytoplasmic fluorescence in the hepatocytes of eight of 12 patients. Specificity testing of these antiserums confirmed that hepatitis B viral markers are present in the hepatocytes of these anti-HBs-positive patients.

These histologic derangements are probably related to frequent treatment with blood products obtained from multiple donors and to the persistence of hepatitis B virus in hepatocytes despite the presence of circulating anti-HBs. (N Engl J Med 298:1373-1378, 1978)

THE high incidence of acute post-transfusion hepatitis with jaundice in hemophilic patients who have received many transfusions has been recognized for many years and was noted to rise with increased use of factor VIII and IX concentrates prepared from pools of 5000 to 10,000 donor plasmas.[1,2] In essentially all patients exposed to factor VIII or IX concentrates either anti-HBs or chronic antigenemia (HBsAg) develops.[3] Of recent concern is the alarmingly high frequency of abnormal liver-function tests, in particular serum glutamic pyruvate transaminase* and glutamic oxalacetic transaminase† observed in these patients.[3-11]

One hundred and twenty patients with hemophilia followed in Pittsburgh have been treated with concentrated factor preparations and have been evaluated with liver-function tests and tests for HBsAg and anti-HBs. The majority were observed for three years or longer. Thirteen (11 per cent) were chronically HBsAg-positive. All but one of the others (88 per cent) were consistently positive for anti-HBs. Forty per cent of the antibody-positive group have had persistently abnormal alanine aminotransferase; all but one have been asymptomatic. To elucidate the importance of these elevations of aminotransferase activity, we studied liver biopsies from 13 anti-HBs-positive, asymptomatic hemophilic patients who were deficient in factor VIII or IX.

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*Now referred to as alanine aminotransferase.

†Now referred to as aspartate aminotransferase.

## MATERIALS AND METHODS

We evaluated 120 hemophilic patients exposed to either factor VIII (Abbott, Armour, Cutter, Hyland or Parke, Davis) or factor IX (Cutter or Hyland) concentrates on at least two occasions at least six months apart and at least 12 months after an episode of clinical hepatitis. Aminotransferases, bilirubin, either alkaline phosphatase or gamma glutamyl transpeptidase, HBsAg (Aus RIA II — Abbott) and anti-HBs (Aus Ab — Abbott) were tested at each visit. Anti-HBc (Cor Ab — Abbott) was tested in 65 of the 120. The mean number of visits per patient was 6.1. The period of observation varied from one to four years.

Forty per cent of the patients in the anti-HBs-positive group have had persistently abnormal alanine aminotransferase. Candidates for biopsy were drawn from this group. After approval for the project had been obtained from the University Human Research Committee, a subset of the patients with abnormal results on liver-function tests who were considered to be co-operative and responsible underwent closed liver biopsies. The extent of abnormality of alanine aminotransferase was not a selection criterion. Biopsies were performed in 10 patients once and in one (Case 8) twice. One (Case 3) had factor-IX-deficient hemophilia. Four (Cases 3, 5, 7 and 9) gave histories of clinical hepatitis with jaundice at least three years before the biopsy. The only other symptoms suggestive of liver disease (Case 11) involved an episode of easy fatigability and alcohol intolerance nine months before biopsy; however, he was clinically well for longer than six months before the procedure. No patient had hepatomegaly or splenomegaly. All biopsies were performed in the Clinical Research Unit, Presbyterian-University Hospital.

Each patient was admitted to the Clinical Research Unit on day

0, a brief history and an informed, signed consent were obtained, and physical examination was performed by one of us (J.A.S.). On day 1 at 8 a.m. an intravenous line was placed with a 19-gauge infusion set (Jelco) from which all subsequent samples were obtained. Blood samples were drawn for determination of alanine aminotransferase, HBsAg, anti-HBs and anti-HBc. Finally, we obtained a blood sample for HLA tissue typing from each patient.

Immediately after the first blood sample the patient received factor VIII concentrate in a dose calculated to raise the serum level to 1 U per milliliter (or to higher than 0.5 U per milliliter for Case 3, who was deficient in factor IX). Twenty minutes later the platelet count, prothrombin time, activated partial thromboplastin time and factor VIII or IX level were determined. All patients showed satisfactory results (platelet count higher than 100,000 per microliter, prothrombin time not more than three seconds longer than control, and factor VIII level approximately 1 U or factor IX level approximately 0.5 U per milliliter) before the biopsy was performed at the bedside with the Klatskin variant of a Menghini needle with a transthoracic approach. On each occasion a second aspiration and on one occasion a third was necessary to obtain an adequate specimen. Routine post-biopsy precautions included frequent vital signs for two hours and complete bed rest for eight hours. The factor VIII or IX level was determined at 2 p.m., and additional factor VIII or IX replacement was given four hours later to raise the level again to a calculated 1 U per milliliter for patients deficient in factor VIII or to 0.5 U per milliliter for the patient deficient in factor IX. On day 2, at 8:30 a.m., factor VIII or IX was administered again to the same calculated level. The mean factor VIII level before biopsy was 0.99 U per milliliter. The pre-biopsy factor IX level in Case 3 was 0.89 U per milliliter. The mean nadir level approximately 22 hours after biopsy (14 hours after a preceding dose) was 0.50 U per milliliter for the patients deficient in factor VIII and 0.25 U per milliliter for the one deficient in factor IX. There was no evidence of bleeding. No alterations were noted in vital signs before and after biopsy. The pre-biopsy mean packed-cell volume was 45.0 and 24 hours after biopsy, 43.7.

Anti-HBc assays were kindly performed by Dr. Harvey Alter, National Institutes of Health Blood Center, Bethesda, Maryland, or by Abbott Laboratories, a solid-phase radioimmunoassay developed by Abbott Laboratory being used.[12] This assay involved a sandwich technic in which the test serum was added to beads containing HBcAg on their surface, and the results reported as the amount of inhibition of $^{125}$I-labeled anti-HBc caused by the test serum. More than 50 per cent inhibition was considered to be positive for anti-HBc in the test sample. Titers were not determined.

The biopsy samples were divided into two portions. One part was sent to the surgical pathology laboratory for sectioning and histologic evaluation. Case 4, a young man with hemophilia B and a history of jaundice in the remote past, had a needle biopsy of the liver performed at the time of cholecystectomy for recurrent cholecystitis. This specimen was processed in the same manner as those obtained by percutaneous biopsy. Liver-biopsy morphology was also available for a patient (Case 13) who fulfilled study criteria and in whom an open biopsy had confirmed Stage 1A Hodgkin's disease. Each biopsy was coded and read independently by one of us (D.H.V.T.) and by a member of the Department of Surgical Pathology. The sections were graded for the presence and amount of fat, fibrosis, polymorphonuclear inflammation, hepatocellular necrosis, Kupffer-cell reactivity, portal inflammation, cholestasis and presence or absence of normal hepatic architecture. The following criteria for pathological diagnoses were used: *chronic persistent hepatitis* — mononuclear infiltrate limited to portal areas by a well defined limiting plate of hepatocytes, with rare foci of chronic inflammatory cells in the lobule; *portal fibrosis* — increased connective tissue limited to portal areas, with no evidence of active hepatocellular injury and with the lobular architecture maintained (a mononuclear infiltrate might or might not be present in the portal areas); *fatty liver* — ballooned hepatocytes with lipid-filled cytoplasm often displacing the hepatocyte nucleus to the periphery of the cell and involving 30 per cent or more of the lobule; *acute hepatitis* — focal hepatocellular dropout and necrosis, with ballooning and Councilman bodies, Kupffer-cell hyperplasia and a mononuclear-cell infiltrate in the portal areas; *subacute hepatitis with bridging necrosis* — same as acute hepatitis, but with areas of necrosis that had become confluent and connected portal areas to adjacent portal areas and to central lobular areas; *chronic active hepatitis* — active necrosis of hepatocytes throughout the lobule, but most marked at the periphery with disruption of limiting plate, extending across multiple lobules and in areas producing a picture of bridging necrosis (mononuclear infiltrates were present within the lobule and portal areas); and *cirrhosis* — loss of normal hepatic architecture with portalization of central veins, hepatocyte regeneration, with double and triple plates common, and nodule formation and fibrosis present.

The second portion of the biopsy (in Cases 1 to 12) was frozen in liquid nitrogen for indirect immunofluorescence studies utilizing two different antiserums. One was nonadsorbed hemophilic serum containing both anti-HBs and anti-HBc. The anti-HBs precipitated with both HBsAg subtypes, ay and ad. The second antiserum, containing human anti-HBc, was kindly provided by Dr. Baruch Blumberg. The anti-HBc in the two antiserums produced comparable amounts of inhibition in the radioimmunoassay (93 per cent by the former and 95 per cent by the latter). No anti-HBs was present in the second antiserum. Neither antiserum was titered, and neither was considered to be monospecific. Each antiserum was diluted 1:10 in phosphate-buffered saline, pH 7.4, and incubated with the tissue for 30 minutes. After washing, a fluorescein-labeled goat anti-human IgG serum was added to the tissue for a subsequent 30-minute incubation. After washing again, the tissue was viewed in an ultraviolet microscope.

Negative controls included incubation of the tissue directly with the fluorescein-labeled anti-IgG serum alone, incubation with the anti-viral serum followed by fluorescein-labeled anti-fibrinogen, and blocking of specific fluorescence by incubation of the tissue with the respective anti-viral serum and then with an unlabeled anti-IgG serum before the conjugate was applied. As a final negative control, each of the previous steps was repeated on normal livers. Marked fluorescence with both antiserums when applied to the livers of HBsAg-positive patients served as a positive control.

Specificity was established by the use of heterologous antiserums assumed to contain no anti-non-A, non-B antibody. Two procedures were performed. In the first, rhesus antiserum specific for HBcAg (kindly provided by Lacy R. Overby, Ph.D., director of experimental biology, Abbott Laboratories) was incubated with cryostat sections of liver from Cases 8b and 10. After washing, the tissue was incubated with a fluorescein-labeled anti-human IgG serum. This antiserum cross-reacted with the rhesus IgG. The resultant labeling pattern was the same as that seen with the serum provided by Dr. Blumberg. In addition, the liver tissue was incubated with the rhesus anti-HBc, after which an unlabeled goat anti-human IgG serum was reacted with the tissue. The tissue was then incubated with the Blumberg serum and then with fluorescein-labeled anti-human IgG. If the two antiserums reacted with the same antigen (presumably HBcAg), labeling of the tissue would be blocked.

For the second procedure a guinea-pig antiserum specific for HBsAg (ad) was obtained from the Research Resources Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland. When this serum was reacted with liver tissue, and the tissue then incubated with a fluorescein-labeled goat anti-guinea-pig IgG, there was labeling in the cytoplasm of hepatocytes. To determine that the original anti-HBs serum was reacting with the same antigen as the specific guinea-pig anti-HBs serum a blocking experiment was done. The tissue was first incubated with the guinea-pig anti-HBs, after which the human anti-HBs and a fluorescein-labeled goat anti-human IgG serum were applied.

## Results

### Serologic Findings

Anti-HBc was present on radioimmunoassay in 86 per cent of the hemophilic patients tested (56 of 65) and in 10 of the 13 on whom biopsies were performed (see Table 1). HLA typing showed HLA-8 to be present in 36 per cent (four of 11) and HLA-9 in 45 per cent (five of 11).

Table 1. Correlation of Histologic, Serologic and Immunofluorescence Data.

| Case No. | Histologic Diagnosis* | SGPT† | Anti-HBs | Anti-HBc | Cytoplasmic Fluorescence‡ | Nuclear Fluorescence‡ |
|---|---|---|---|---|---|---|
| 1 | CPH | 98 | +§ | 0 | 0/0 | 0/0 |
| 2 | CPH | 107 | +§ | 0 | 0/0 | 0/0 |
| 3 | CPH | 82 | +§ | + | 0/0 | 0/0 |
| 4 | CPH | 39 | + | + | 0/0 | 0/0 |
| 5 | CPH | 152 | + | 0 | +/0 | +/0 |
| 6 | CPH | 88 | + | + | +/0 | +/0 |
| 7 | CPH | 52 | +§ | + | +/0 | +/0 |
| 8a | AH | 127 | + | + | ND¶/0 | ND/0 |
| 8b | CAH | 114 | + | + | +/+ | +/+ |
| 9 | CAH | 33 | +§ | + | +/+ | +/+ |
| 10 | CAH | 295 | +§ | + | +/+ | +/+ |
| 11 | CAH + PNC | 53 | +§ | + | +/+ | +/+ |
| 12 | FL + PF | 80 | + | + | +/0 | +/0 |
| 13 | CPH | 86 | + | + | ND | ND |

*CPH denotes chronic persistent hepatitis, AH acute hepatitis, CAH chronic active hepatitis, PNC postnecrotic cirrhosis, FL fatty liver, & PF portal fibrosis.
†Serum alanine amino transferase (normal <25 IU).
‡See text. §Ratio of patient counts/control >100. ¶Not determined.

### Liver-Biopsy Morphology

Six patients (Cases 1-3 and 5-7) of the biopsied percutaneously were classified as having chronic persistent hepatitis (Table 1). The two who underwent open liver biopsies (Cases 4 and 13) also showed chronic persistent hepatitis. One patient (Case 8) appeared to have acute hepatitis, although there had been no recent alteration in his clinically asymptomatic state or in alanine aminotransferase. Figure 1 shows this biopsy as well as a second performed 13 months later demonstrating chronic active hepatitis. Cases 9 and 10 had chronic active hepatitis, as did Case 11, who also had postnecrotic cirrhosis (this patient was the one with a past history of symptoms suggestive of liver dysfunction). Case 12 was found to have fatty infiltration with advanced portal fibrosis, although he gave no history of very excessive alcohol consumption.

### Liver Biopsy — Immunofluorescence

Using an indirect technic (Table 1), we found fluorescence in both the cytoplasm and the nucleus of eight of 12 patients with the hemophilic antiserum (shown before the slash) and in four of 12 with the Blumberg antiserum (shown after the slash). The nuclear fluorescence was speckled, consisted of variable numbers of particles per nucleus and was seen only in hepatocytes. Aggregates of three to four hepatocytes showing this staining pattern were found scattered throughout the biopsy. Approximately 10 per cent of the hepatocytes had positive nuclear fluorescence. The cytoplasmic fluorescence was perinuclear and usually clumped to one side of the nucleus.

The specificity of the antiserums was evaluated in two patients (Cases 8b and 10). The liver of each patient showed cytoplasmic fluorescence with use of guinea-pig anti-HBs and nuclear fluorescence with rhesus anti-HBc. When blocking studies using the guinea-pig anti-HBs before addition of the hemophilic antiserum were performed, cytoplasmic fluorescence was obliterated. Blocking of nuclear fluorescence was observed when rhesus anti-HBc was added before either the hemophilic or the Blumberg antiserum.

### Correlation of Histologic and Serologic Findings and Immunofluorescence

Table 1 lists each patient's histologic diagnosis in approximate order of morphologic abnormality, serum alanine aminotransferase, anti-HBs and anti-HBc, as well as the demonstration of cytoplasmic and nuclear fluorescence within liver cells. The asterisk denotes high-ratio anti-HBs in the serum (>100 times control). It is apparent that there was little relation between the level of alanine aminotransferase and the extent of histologic abnormality. Moreover, there was no relation between the presence of high-ratio anti-HBs and the development of severe liver disease. Finally, cytoplasmic and nuclear fluorescence was demonstrated in the patients with the most morbid histologic disease.

### Liver Histologic Findings, Immunofluorescence and Fraction Exposure

The histologic status of the liver was compared with the age of the patient, treatment in units of factor VIII or IX concentrate received in the past year, treatment with factor VIII or IX concentrate during the patient's lifetime and the presence of nuclear fluorescence. Little correlation was evident.

## Discussion

Hemophilic patients represent an uncommon group in that, unlike the usual victims of transfusion-induced hepatitis, they are probably repeatedly exposed to low doses of hepatitis B virus as well as other blood-borne viruses — e.g., non-A, non-B hepatitis virus (or viruses)[13,14] and, thus, development of a chronic process may be more likely. In addition, the potential persistent presence of viral-anti-viral immune complexes in these patients may alter many of the criteria currently associated with the diagnosis of chronic post-transfusion hepatitis. These considerations, in addition to uncertainty about the clinical implications of persistently abnormal results on liver-function tests, with no trend toward worsening or improvement, prompted the present study. Liver biopsies in patients with hemophilia require, for safety, large doses of the coagulant concentrates and, hence, are expensive and should be confined to knowledgeable centers. However, when guidelines for the treatment of asymptomatic liver disease are better defined, the information gained from such biopsies should be very important to the hemophilic patient's future welfare.

To date, noninvasive clues to the severity of morphologic liver disease have been less than satisfactory. Serum alanine aminotransferase levels have been

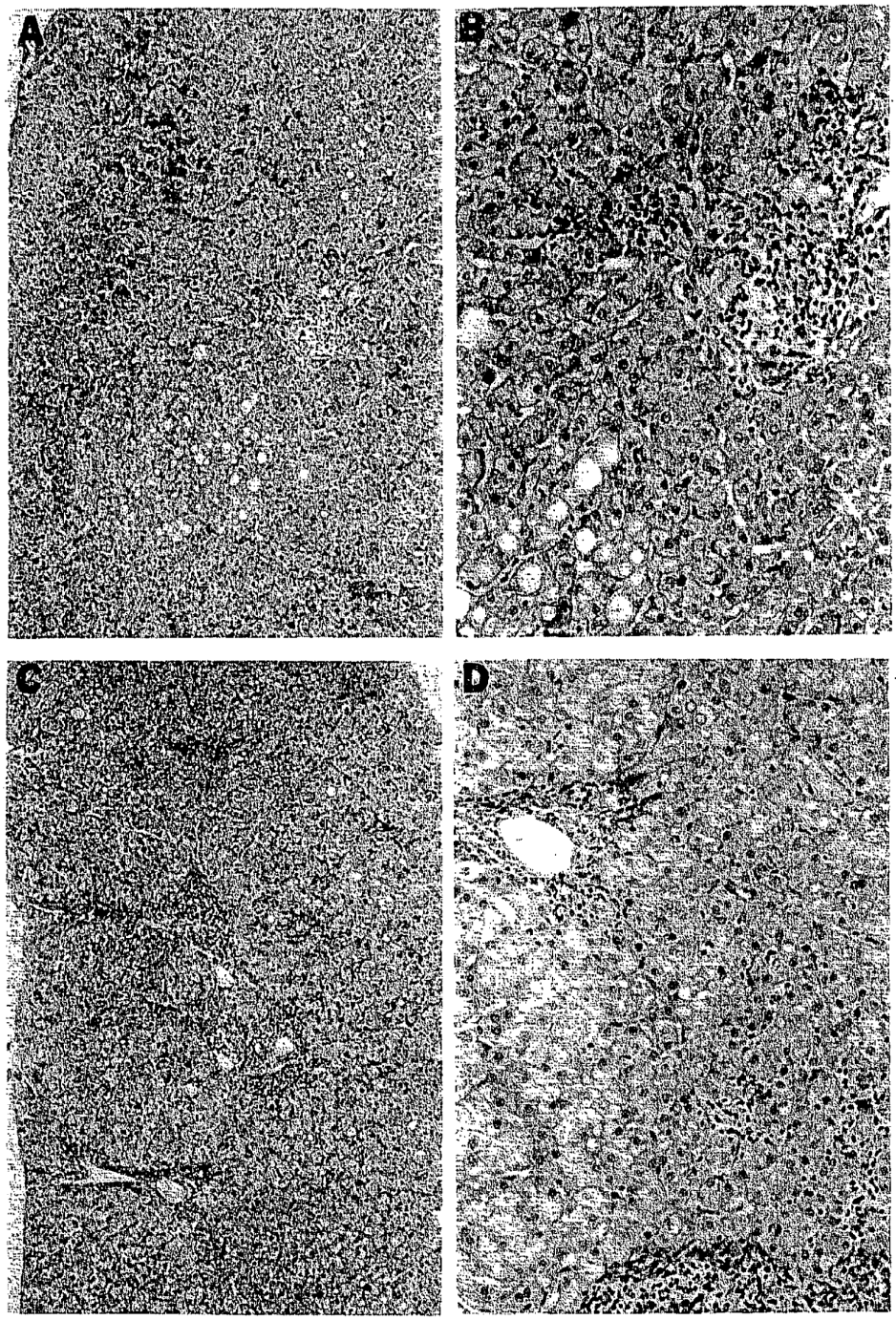


Figure 1. Photomicrographs of the Two Liver Biopsies Obtained from Case 8.
A and B are low (× 40) and high (× 125) magnifications of the first biopsy, and C and D low and high magnifications of the second biopsy. Note the development of dense portal infiltrates with poorly defined limiting plates in D as compared to B.

reported not to be predictive,[15] and this lack of correlation was confirmed in the present study. A higher than normal frequency of the tissue histocompatibility antigen, HLA-8,[16,17] in liver disease was not supported by this or other studies.[18] In the biopsied group HLA-8 was observed in 36 per cent, and HLA-A9 in 45 per cent. This frequency compares to 29 and 15 to 20 per cent, respectively, reported in healthy controls and in patients with a variety of liver disorders.[18] Only one of the four patients with chronic active hepatitis had HLA-8. In addition, the present study showed no apparent correlation between the titer of anti-HBs (Table 1) and the severity of liver-biopsy findings. Although a high ratio of anti-HBs may be protective against acute hepatitis caused by hepatitis B virus,[3] its presence appears to have no effect on the development of chronic liver disease in hemophilia. Furuta[19] and Kojima[20] and their colleagues, using an immune adherence hemagglutination assay, and Hoofnagle and his associates,[21] on the basis of a complement-fixation assay, have suggested that high-titer anti-HBc may be associated with active, rather than remote, infection, and others have reported a possible association with oncogenicity.[22] Using a non-titered radioimmunoassay, we found anti-HBc in the serum of 85 per cent of the hemophilic patients studied and in 10 of the 13 biopsied. Of interest is its absence only in those with the most favorable histologic findings. The presence of anti-HBc in the serum of 85 per cent of these patients, who had received many transfusions, is much more frequent than previous studies have reported — 26 per cent, with use of a complement fixation.[21] This finding is probably related to the observation by Grady et al.[23] that the radioimmunoassay for anti-HBc was significantly more sensitive than complement fixation.

In the present study a total of 14 liver biopsies in asymptomatic patients with hemophilia were reviewed. This procedure included a follow-up biopsy at 13 months on one of these patients. All demonstrated histologic liver disease. Eight (including the two open biopsies) showed a relatively benign form of pathologic change — i.e., chronic persistent hepatitis — whereas five patients had severe liver disease. For the present, hemophilic patients who are asymptomatic and have chronic active hepatitis on biopsy have not been begun on corticosteroid therapy because of its potential hazards and the lack of knowledge of the natural course of the disorder in clinically well persons. Lesesne et al.[11] biopsied six patients with hemophilia (of whom two were both HBsAg positive and anti-HBs positive, and four anti-HBs positive only). In the former group, both had chronic active hepatitis, and in the latter, one had chronic active hepatitis and the remaining three, chronic persistent hepatitis. Five of their six patients had symptomatic disease and were subsequently treated with corticosteroids. Two additional studies evaluated results of liver biopsy in hemophilia, but neither attempted a systematic discrimination between groups that were HBs antigen or antibody positive or on the basis of presence or absence of symptoms.[24,25]

The demonstration of HBsAg and HBcAg in the livers of anti-HBs-positive hemophilic patients by indirect immunofluorescence with serum containing anti-HBs and anti-HBc was an unexpected finding. Since the early 1970's much has been learned about hepatic immunofluorescence with anti-HBs and anti-HBc. Almeida et al.[26] demonstrated a differentiation in nuclear versus cytoplasmic fluorescence that was dependent upon the antibody used (anti-HBc or anti-HBs); they showed that nonadsorbed hemophilic serum fluoresced both nucleus and cytoplasm. Ray et al.[27] and other authors[28,29] have confirmed the relative specificity of HBcAg for the nucleus and HBsAg for the cytoplasm. Although the large majority of studies using such technics have focused on serologically antigen (HBsAg)-positive patients, two studies[20,27] have demonstrated HBsAg and HBcAg in hepatocytes from persons serologically negative for HBsAg on radioimmunoassay. However, their anti-HBs status was not documented. Thus, the presence of HBsAg and HBcAg in the livers of these anti-HBs-positive hemophilic patients is unusual.

Although the antiserums that we used were not monospecific, their specificity was clarified by further studies. The hemophilic antiserum contained both anti-HBs and anti-HBc; in addition, it is possible that both antiserums contained antibody to non-A, non-B hepatitis producing virus (or viruses) that at present cannot be further identified. However, the finding of similar fluorescence patterns in the two patients tested (Cases 8b and 10) by use of guinea-pig anti-HBs and rhesus anti-HBc, both of which should be free of anti-non-A, non-B, and the ability of these antiserums to block fluorescence produced by the hemophilic and Blumberg antiserums strongly suggests that both HBsAg and HBcAg are present in the hepatocytes of the anti-HBs-positive hemophilic patients who showed fluorescence. The presence of cytoplasmic staining with the Blumberg antiserum (anti-HBc) is somewhat unusual, but such non-nuclear fluorescence with a similar antiserum with use of a direct fluorescence technic has previously been reported and has been interpreted as representing intra-cytoplasmic HBcAg.[27] The failure of the Blumberg antiserum to produce fluorescence in as many livers as the hemophilic antiserum (four livers with the former and eight with the latter) is disturbing but may be explained on the basis of antigenic heterogeneity of HBcAg, resulting in varying interaction with the two, different anti-HBc antiserums. Nevertheless, the finding of positive fluorescence in liver biopsies obtained from these asymptomatic anti-HBs-positive hemophilic patients suggests that either intact virus or viral products are present in their livers in the absence of a clinical picture consistent with active hepatitis B infection. Whether these viral products contribute to the development or perpetuation of chronic progressive liver disease cannot be determined at present.

It is interesting to speculate on the cause of the persistence of anti-HBs in hemophilic patients. Is it due to possible endogenous antigen in the liver or, as commonly believed, to repeated exposure to exogenous antigen — e.g., the concentrates? Several patients followed in Pittsburgh have received concentrates on only one occasion before 1975 and remain anti-HBs positive, with abnormal serum alanine aminotransferase three years later. Perhaps a chronic process reacting to hepatitis B virus persisting in the hepatocytes perpetuates this antibody response and produces liver damage. There are few liver-biopsy data on subjects who are anti-HBs positive but have no suggestion or past history of liver disease. In the one available study, Iwarson et al.[30] have demonstrated normal hepatic morphology in a small group of anti-HBs-positive blood donors with normal results in liver-function tests. However, immunofluorescence was not studied.

Our results suggest that, throughout the world, a large number of asymptomatic hemophilic patients who have received numerous transfusions must have histologic liver disease. In some, it must be severe. Adequate information is not yet available to evaluate fully hemophilic patients treated only with blood products negative by radioimmunoassay for HBsAg in both donors and final product. However, at least two patient cared for in Pittsburgh and treated only with such concentrates have had persistent, biochemical abnormalities, and one has remained HBsAg positive for more than one year. In addition, the implications of non-A, non-B virus (or viruses) in hemophilia have yet to be evaluated. To return to less effective therapy for hemophilic bleeding would represent a step backward. It is apparent, then, that a major effort is necessary to develop a "clean" product rapidly for the treatment of the next generation of hemophilic patients.

Finally, although this study was performed on patients with hemophilia, the finding of HBsAg and HBcAg in the livers of serologically anti-HBs-positive persons has a far broader implication. It opens to question the prevailing concept of the protective nature of anti-HBs. Perhaps further study of this unusual group of patients will clarify the questions raised.

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