the disease until attacks can be very accurately predicted, or the natural history substantially changed.

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CLINICAL AND SEROLOGICAL ANALYSIS OF TRANSFUSION-ASSOCIATED HEPATITIS

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Summary Of 108 prospectively followed, multiply transfused, open-heart-surgery patients, 12 (11%) developed hepatitis. Patients received only volunteer donor blood tested for hepatitis-B surface antigen (HBsAg) prior to transfusion by counterelectrophoresis (C.E.P.). 4 of the 12 patients developed hepatitis-B-virus infection. Subsequent testing of donor serums by solid-phase radioimmunoassay (R.I.A.) revealed that an R.I.A.-positive, C.E.P.-negative blood unit was transfused to 3 of the 4 type-B hepatitis cases, but to none of the remaining 104 patients; 3 hepatitis-B cases could probably have been prevented by prescreening of donors by solid-phase R.I.A. 8 hepatitis cases were serologically unrelated to the hepatitis-B virus, the hepatitis-A virus, the cytomegalovirus, or the Epstein-Barr virus. Had R.I.A.-positive donors been excluded, 8 of the 9 residual hepatitis cases (89%) would have represented "non-A, non-B" hepatitis. The existence of previously unrecognised human hepatitis virus(es) is probable.

Introduction

THE attainment of hepatitis-free blood-transfusions has been a frustratingly slow, but progressively realistic goal. The demonstration of the inordinately high hepatitis risk of commercial blood[1,2] and the implementation of universal donor screening for hepatitis-B surface antigen (HBsAg) have resulted in a distinct reduction in post-transfusion hepatitis.[3,4] Nonetheless, transfusion-associated hepatitis continues to occur. This study examines the potential impact of solid-phase radioimmunoassay ('Ausria') on the frequency of type-B post-transfusion hepatitis and the relative role of agents other than the hepatitis-B virus in the causation of transfusion-associated hepatitis.

Patients and Methods

Design of Study

Consecutive patients undergoing open-heart surgery at the National Institutes of Health were entered into the study if they were over 21 years of age, if they lived in the continental United States, if pre-operative S.G.P.T. was normal, and if they had not had transfusions or known hepatitis exposure during the six months preceding surgery. 106 patients fulfilled these criteria and completed six months of clinical and serological observation as defined below. 2 of these 106 patients were operated on twice during the study period at intervals exceeding six months, and, for purposes of analysis, it will henceforth be considered that 108 patients were studied.

Eligible patients were divided into two groups according to their geographic location as previously described.[3] Local patients (47) had samples drawn approximately weekly for the first twelve weeks post-transfusion and monthly for three additional months; the average collection per patient was 17·7 samples. Patients (61) who lived at a great distance from N.I.H. had samples drawn by their referring physicians and the separated serum was immediately mailed to N.I.H. In this group, blood-samples following hospital discharge were obtained every two weeks during the first twelve postoperative weeks and then monthly for the succeeding three months; the average number of samples was 13·5. If enzyme abnormalities in either patient group indicated the onset of hepatitis, samples were obtained weekly until the acute hepatitis resolved and at varying intervals thereafter. All patients who developed hepatitis had samples obtained for at least a year in order better to ascertain antibody seroconversion.

Only voluntary donor blood was used throughout this study. All donor blood was tested for hepatitis-B surface antigen (HBsAg) by counterelectrophoresis prior to transfusion; all donor serums were subsequently retested by solid-phase radioimmunoassay after transfusion. When it became apparent that radioimmunoassay might have prevented several cases of type-B post-transfusion hepatitis which occurred in this study, and when it became technically feasible to incorporate the radioimmunoassay into pretransfusion donor screening, the study was terminated.

Definitions

Hepatitis.—A patient was considered to have post-transfusion hepatitis when, between fourteen and a hundred and

eighty days after transfusion the alanine-aminotransferase (S.G.P.T.) level rose to 2·5 times the upper limit of normal (112 I.U./l) and when a second sample, separated by at least a week, exceeded two times the upper limit of normal (90 I.U./l). Enzyme values and clinical histories were reviewed by a panel* and the diagnosis of viral hepatitis accepted only when other causes of enzyme elevations such as congestive failure and drug or anæsthesia-induced hepatitis could be reasonably excluded No evidence was found in any patient to suggest that the hepatitis was contracted from sources other than transfusion. Icteric hepatitis was diagnosed when the serum-bilirubin exceeded 2·0 mg/dl.

Antibody response.—Antibody response consisted of either a fourfold or greater rise in titre in a patient who had pre-existing antibody or of antibody seroconversion. Seroconversion was defined as the *de-novo* appearance of antibody two or more weeks post-transfusion in a patient whose pre-transfusion sample was negative for antibody.

Test Methods

Alanine-aminotransferase (S.G.P.T.) and aspartate-aminotransferase (S.G.O.T.) determinations were performed by the Clinical Chemistry Laboratory at N.I.H. using the method of Steinberg et al.[5] and Wroblewski,[6] respectively. The upper limit of normal for S.G.P.T. was 45 I.U./l and for S.G.O.T. 52 I.U./l.

Counterelectrophoresis (C.I.P.) on donor serum was performed by 'Hepascreen' (Spectra Laboratories) according to the directions of the manufacturer.

Solid-phase radioimmunoassay for HBsAg was performed on all sera using the method of Ling and Overby (Ausria I).[7] Sera from patients who developed hepatitis and from donors implicated in hepatitis cases were also tested subsequently by the more sensitive and more specific Ausria II. All positive results were confirmed by repeat testing and by neutralisation with unlabelled human antibody to HBsAg (anti-HBs). A test was considered positive if radioactive counts exceeded the mean of eight negative controls by a factor of at least 2·1 and if prior neutralisation with unlabelled anti-HBs reduced radioactive counts by at least 50% while neutralisation with normal human serum had no significant effect.

Subtyping of HBsAg was done by the counterelectrophoretic technique of Holland and associates.[8]

Hæmagglutination tests for anti-HBs were performed on all patient samples by the method of Vyas and Shulman[9] using a microtitre system and HBsAg-coated red cells obtained from Electronucleonics, Inc., Bethesda, Maryland. A titre of 1/8 or greater was considered to demonstrate the presence of anti-HBs. At the completion of the study, all serial serum samples from any one patient who had an anti-HBs response were retested on the same day using the same set of HBsAg-coated and control red cells.

Radioimmune precipitation for anti-HBs was performed on a pre-transfusion and a six-to-nine-month post-transfusion sample from each patient by a double-antibody microtitre technique;[10] binding of ^{125}I-labelled HBsAg of greater than 15% on duplicate analysis was considered positive. Antibody to the hepatitis-B core antigen (anti-HBc) was measured in a pre-transfusion and a three and six month post-transfusion sample in all patients by the radioimmune precipitation technique of Moritsugu et al.;[11] a titre of 1/1000 or a fivefold or greater rise in titre was required for a sample to be regarded as unequivocally containing anti-core antibody acquired by recent infection with the hepatitis-B virus.

Pre-exposure and convalescent sera from patients with hepatitis were tested for antibody to the hepatitis-A virus (anti-H.A.), to the cytomegalovirus (anti-C.M.V.), and to the Epstein-Barr virus (anti-E.B.V.). The presence of antibody to the hepatitis-A virus was determined by immune-electron microscopy (I.E.M.) employing the method of Feinstone et al.[12] and by immune adherence.[13] In the I.E.M. test, paired sera from the same patient were always tested under code in the same experiment and against the same particle-containing stool filtrate. Based on the reproducibility of scoring coded specimens, a change of 1 (scale 0–4) in antibody rating between paired sera was considered significant. A serum sample was not considered negative for antibody until at least five good-quality electron microscopic grid squares were examined. Immune adherence C.I.A. was performed with hepatitis-A antigen partially purified from fæces of acutely ill patients with hepatitis type A (Moritsugu et al., unpublished). Twofold dilutions of serum samples were tested against 4–8 units of antigen. Hæmagglutination was scored on a scale of 0–4+; 3+ and 4+ patterns were regarded as positive.

Complement-fixation tests for anti-C.M.V. were performed as previously described.[14] C.M.V. antigen was prepared from the AD 169 strain of human C.M.V.[15]

Paired sera were titrated in duplicate for anti-E.B.V. by immunofluorescence[16] using HRI-K cells as a source of antigen. Serum samples obtained during the acute phase of hepatitis were tested for hepatitis-B-specific D.N.A. polymerase by the method of Kaplan and coworkers.[17]

Results

Frequency of Hepatitis

Of the 108 patients 12 (11%) developed hepatitis; 4 of the 12 cases were icteric (3·7% of total patients). The average number of transfusions was 17·3 units per patient resulting in a hepatitis risk of 6·4 cases/1000 units transfused (0·64% per unit) and an icteric hepatitis risk of 2·1 cases/1000 units transfused (0·21% per unit).

Serological Analysis of Hepatitis Cases

Table I records the serological evaluation of the 12 hepatitis cases. As listed in the table, the first 4 cases have been classified as viral hepatitis, type B: all 4 developed HBsAg in the course of their acute hepatitis and 3 of the 4 demonstrated antibody seroconversion for both hepatitis-B surface and core antigens. Patient no. 4 developed anti-HBc, but not anti-HBs; she has become a chronic carrier of HBsAg. 2 of the hepatitis-B cases (nos. 2 and 4) were of subtype *ayw* and 1 (no. 1) was *adw*. The titre of HBsAg was not sufficiently high in patient no. 3 to determine the HBsAg subtype. The serological specificity in cases 1 and 2 was confirmed by the development of hepatitis-B-specific D.N.A. poly-

TABLE I—HEPATITIS CASES: SEROLOGICAL ANALYSIS

Patients no.	HBsAg Positive	Serological response* to:				
		HBsAg	HBcAg	C.M.V	E.B.V.	H.A.V.
1	Yes	Yes	Yes	No	No	No
2	Yes	Yes	Yes	No	No	No
3	Yes	Yes	Yes	No	No	No
4	Yes	No†	Yes	No	No	No
5	No	No	No	No	No	No
6	No	No	No	No	No	No
7	No	No	No	No	No	No
8	No	No	No	No	No	No
9	No	No	No	Yes‡	No	No
10	No	No	No	No	No	No
11	No	No	No	Yes‡	No	No
12	No	No	No	No	No	No

* Antibody seroconversion or fourfold or greater rise in antibody titre to the hepatitis-B surface antigen (HBsAg), hepatitis-B core antigen (HBcAg), cytomegalovirus (C.M.V.), Epstein-Barr virus (E.B.V.) or the hepatitis-A virus (H.A.V.).

† Became chronic carrier of HBsAg.

‡ 5 of 26 control patients without hepatitis also made a serological response to C.M.V.

* We are indebted to Dr Leonard Seeff for serving on this panel along with H.J.A., P.V.H., and R.H.P.

TABLE II—HEPATITIS CASES: CLINICAL ANALYSIS

Hepatitis type	No. of cases	Incub. period (wk.)*		Peak S.G.P.T. (I.U./l)		Total icteric	Peak bilirubin Mean (range)	No. chronic hepatitis (>6 mos.)
		Mean	Range	Mean	Range			
B	4	14·5	8–23	857	438–1300	3	7·8 (0·6–24)	1†
Non-B	8	9·4	6–22	470	185–1200	1	1·3 (0·4–5·3)	1†

* From transfusion to first S.G.P.T. greater than 2·5 times normal.
† Each demonstrated chronic active hepatitis by liver biopsy.

merase concurrently with the development of HBsAg. Patients 3 and 4 did not have detectable hepatitis-B D.N.A. polymerase activity. None of the type-B hepatitis cases demonstrated antibody seroconversion to C.M.V., E.B.V., or hepatitis-A virus.

The remaining 8 cases listed in table I were considered to represent non-B hepatitis: none developed HBsAg, anti-HBs, or anti-HBc. Each of the non-B hepatitis cases was tested for antibody response to C.M.V., E.B.V., and the hepatitis-A virus. 1 of the 8 demonstrated antibody seroconversion to C.M.V. and a 2nd showed a four-fold rise in titre. However, antibody seroconversion to C.M.V. was also seen in 5 of 26 controls who did not develop hepatitis. Each of the 8 non-B-hepatitis patients had antibody to E.B.V. present in their pretransfusion sample and none showed a rise in titre. 6 of the 8 patients had antibody to the hepatitis-A antigen present in their pre-transfusion sample and none showed a rise in antibody activity during or after their hepatitis. Patients 6 and 10 did not have antibody to hepatitis-A antigen in either their pre- or post-transfusion samples. There was agreement between hepatitis-A antibody results obtained by I.E.M. and I.A.

Clinical Analysis of Hepatitis Cases

Table II compares the clinical data obtained in type-B versus non-B hepatitis. The mean incubation period, as measured by the first S.G.P.T. elevation to exceed 2·5 times the upper limit of normal, was approximately five weeks longer in type-B than in non-B hepatitis, but the range was broad in each group and there was much overlap. The period from transfusion to the onset of HBsAg in the 4 type-B cases was four, nine, ten, and fourteen weeks.

The mean peak S.G.P.T. in type-B hepatitis was 1·8 times that in non-B hepatitis, but the range in each group was again broad. S.G.P.T. exceeded ten times the upper limit of normal in 3 of the 4 type-B cases and in 3 of 8 non-B cases. 3 of the 4 type-B hepatitis cases were icteric compared with only 1 of 8 non-B cases, and the mean peak bilirubin in type-B hepatitis was six times that in non-B disease. All 4 patients with type-B hepatitis had clinical symptoms consistent with viral hepatitis, whereas only 2 of 8 patients with non-B hepatitis had symptoms; one of the symptom-free non-B patients, however, progressed to chronic active hepatitis. Excluding the one patient in each group who developed chronic active hepatitis, the mean time for which S.G.P.T. exceeded two times the upper limit of normal was identical in those with type-B hepatitis (10·3 weeks) and those with non-B hepatitis (10·4 weeks).

Retrospective Analysis of Implicated Donors

The 108 patients in this study received 1870 units of C.E.P.-negative, voluntary donor blood. The results of retrospective testing of the donors by radioimmunoassay are shown in table III. Specific R.I.A.-positive, C.E.P.-negative blood was transfused to 3 of the 4 patients who developed type-B hepatitis. In contrast, none of the 8 patients with non-B hepatitis or of the 96 patients without hepatitis received blood which was R.I.A.-positive.

TABLE III—RETROSEPCTIVE ANALYSIS OF DONORS BY SOLID-PHASE R.I.A. FOR HBSAG

Patient outcome	No. of cases	No. of cases with R.I.A.+, C.E.P.-donor
Type-B hepatitis	4	3
Non-B hepatitis	8	0
No hepatitis	96	0

Serological Analysis of Non-hepatitis Cases

Of the 96 patients who did not develop hepatitis, none became HBsAg-positive and none developed antibody seroconversion to HBsAg. 2 patients, in their six-month post-transfusion sample, demonstrated anti-core antibody at the minimum titre considered to represent a positive result. All preceding and subsequent samples in these patients were negative for anti-HBc. 7 patients had anti-HBc prior to transfusion; 5 of these 7 also had anti-HBs, usually in high titre, but in 2 patients anticore antibody was present without coexisting anti-HBs.

Significance of Anti-HBs in Recipient and Donor

14 (13%) of the 108 patients had anti-HBs detectable in their pre-transfusion sample. None of these 14 patients developed type-B hepatitis, compared to 4 of 94 who did not have pre-existing anti-HBs. The apparent protective effect of pre-existing anti-HBs did not, however, achieve statistical significance.

Samples of all donor blood (HBsAg-negative) administered to 36 of the 108 patients were available for anti-HBs testing. Of these 36 patients, 12 received at least one unit of blood containing anti-HBs. None developed HBsAg-positive hepatitis, or a serological response to the hepatitis-B surface or core antigens; 1 developed HBsAg-negative hepatitis.

Discussion

Type-B hepatitis continues to occur following transfusion, despite widespread screening of blood-donors by C.E.P., as demonstrated in this and other studies.[3,18] In the current study, retrospective testing of donor blood by solid-phase radioimmunoassay demonstrated that this test would have been highly effective in reducing type-B hepatitis. 3 of the 4 patients who developed type-B post-transfusion had received one unit of R.I.A.-positive, C.E.P.-negative blood. Conversely, only 3 R.I.A.-positive, C.E.P.-negative units of blood were transfused and each resulted in overt, icteric HBsAg-positive hepatitis, with 1 patient progressing to chronic active hepatitis. It is thus probable that 3 of the 4 type-B hepatitis cases could have been prevented if donor blood had been screened for HBsAg by solid-phase R.I.A. rather than C.E.P.

Two previous publications[19 20] stressed the inability of solid-phase R.I.A. to reduce the frequency of type-B hepatitis. However, both studies were performed at a time when up to 80% of positives by R.I.A. represented false-positive tests,[21 22] and neither study incorporated appropriate specificity testing. Subsequently, modifications in the solid-phase R.I.A. system have reduced non-specificity to under 1% and all positive tests are required to be confirmed by appropriate neutralisation. Hollinger et al.[23] have reported that, despite specificity testing of solid-phase R.I.A., this test was markedly inferior to their own double-antibody radioimmunoassay and relatively ineffective in preventing type-B hepatitis. Not only does this contrast with the efficacy of solid-phase R.I.A. in our study, but the time required and the complexity of double-antibody R.I.A. makes this an impractical test for the routine screening of blood-donors. The increased sensitivity of solid-phase R.I.A. compared with C.E.P. has been repeatedly demonstrated by in-vitro assays.[22–24] The present data demonstrate that this increased in-vitro sensitivity would also be reflected in a decrease in overt type-B post-transfusion hepatitis. It is mandatory that an objective test of comparable sensitivity, specificity, practicality, and clinical efficacy replace the second-generation tests currently employed in some donor facilities. Such "third-generation" testing has recently become a legal requirement in the United States.

The other major contribution of the current study is the clinical and serological evaluation of those hepatitis cases unrelated to the hepatitis-B virus. 8 of the 12 cases which occurred were serologically classified as "non-B" hepatitis. From a clinical standpoint, type-B hepatitis had a longer mean incubation period, but the overlap in incubation periods was so great as to make this a distinction of little diagnostic consequence. The mean incubation period for non-B hepatitis (9·4 weeks) lies between that classically attributed to type A and type B hepatitis and may, as has been suggested,[25 26] provide epidemiological evidence for a third human hepatitis virus. Type-B hepatitis appeared to be more acutely severe than non-B hepatitis according to the mean peak S.G.P.T., the frequency of icterus, and the frequency and severity of symptoms. This is consistent with our previous experience and with another published study.[27] Nonetheless, 3 of the 8 non-B cases had a peak S.G.P.T. in excess of ten times the upper limit of normal and 1 of these patients developed chronic active hepatitis.

Serological analysis of the 8 cases of non-B hepatitis revealed no ætiological relationship to the cytomegalovirus or the Epstein-Barr virus. We had previously assumed that the major portion of transfusion-associated hepatitis unrelated to the hepatitis-B virus was due to the hepatitis-A virus, but this was not substantiated in the present study. Application of both the I.E.M. and immune-adherence techniques demonstrated that none of these 8 cases was serologically related to the hepatitis-A virus. Some of these cases were also included in a larger study of the relationship of the type-A virus to non-B post-transfusion hepatitis.[28] In that retrospective analysis, serum from a total of 22 non-B hepatitis cases were tested and none demonstrated a serological response to the hepatitis-A virus. It thus appears that the hepatitis-A virus is rarely involved in the development of transfusion-associated hepatitis. These serological data suggesting the rarity of type-A post-transfusion hepatitis are compatible with the epidemiological data reported by Prince et al.[26]

The ætiology of non-A, non-B hepatitis after transfusion remains obscure. One could argue that these transaminase elevations do not represent viral hepatitis, but every effort was made to exclude other known causes of hepatic enzyme elevation. The strongest evidence that non-A, non-B hepatitis is a transfusion-related (and, by inference, virus-related) event is the fact that it appears to occur with a defined incubation period and, more important, that, like type-B hepatitis, it is considerably more common after the receipt of commercial blood than of voluntary donor blood.[26] There is thus increasing suspicion that there exists one or more previously unrecognised human hepatitis virus(es). The significance of the postulated virus(es) is emphasised by the fact that, if we eliminate from this study the 3 cases of type-B hepatitis which probably could have been prevented by R.I.A. screening of blood-donors, then 8 of the 9 residual hepatitis cases (89%) could be classified as "non-A, non-B". When blood is obtained from all-voluntary, R.I.A.-screened donor population, the vast majority of resultant hepatitis is currently unrelated to any of the viruses commonly associated with human hepatitis.

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