

AN ANTIGEN DETECTED IN THE BLOOD DURING THE INCUBATION PERIOD OF SERUM HEPATITIS*

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The aim of the following experiments was to provide an objective immunologic criterion for the diagnosis of serum hepatitis, as well as a possible means of screening for carriers of the agent of this disease. An antigen that reacted in the immunodiffusion test with serum from multiply transfused patients was detected in the blood during the incubation period prior to the onset of major chemical or clinical abnormalities. Double blind experiments suggest that this antigen is specific for serum hepatitis virus.

Materials and Methods.—*Clinical specimens:* Sera from cases of transfusion-induced viral hepatitis, which we have collected, were obtained as part of a long-term study involving biweekly follow-up of transfused patients at The New York Hospital. Patients volunteering to participate in this study provided blood samples prior to transfusion and at least biweekly for a period of 6 months or more following transfusion.

Test serum: The reference "antiserum" used in the majority of the studies to be described, hereinafter referred to as serum *S*, was obtained from a 24-year-old male patient with hemophilia who has received more than 10,000 units of blood, fresh-frozen plasma, and cryoprecipitate during the course of treatment for bleeding episodes. He has had no episodes of icteric hepatitis, but it was presumed that he had been multiply exposed to the virus or viruses of serum hepatitis. Serum *S* was chosen for these studies because the patient's multiple exposure was thought to ensure a hyperimmune status. Subsequently, four other sera from multiply transfused patients have been found to react in a manner similar to serum *S*. For some experiments, the serum was concentrated by ethanol fractionation. To each milliliter of serum to be concentrated, 8 ml of 30% ethanol in 0.1 *M* NaCl, 0.01 *M* tris(hydroxymethyl)aminomethane (Tris), 0.001 *M* ethylenediaminetetraacetate (EDTA), (pH 7.0 at -7°C) were added. This mixture was held at -7°C and lyophilized. The dried globulin fraction was then rehydrated with distilled water to 0.1 the original volume of serum employed.

Immunodiffusion technique: Double diffusion in agar gel was done by a micro-Ouchterlony technique.¹ Nonspecific precipitation reactions between adjacent wells were eliminated by the use of 0.9% agarose dissolved in a buffer composed of 0.1 *M* NaCl, 0.01 *M* Tris (pH 7.6 at 25°C), and 0.001 *M* EDTA containing 1 mg/ml protamine sulfate. Protamine sulfate has been recently suggested as a means of decreasing virus-agar interaction.² Plates were incubated in a humid atmosphere at room temperature and read daily for 7 days. Strong reactions were evident after overnight incubation, while weaker reactions required 2 or 3 days' incubation and intensified for several days.

Clinical chemical methods: Serum glutamic pyruvic transaminase (SGPT) was assayed by a kinetic spectrophotometric method with the Gilford multiple method sample recording spectrophotometer.³ Serum lactic dehydrogenase (LDH) enzymes were assayed by the method of Amador *et al.*⁴ with the same instrument. Serum LDH isoenzymes were separated by thin agar gel electrophoresis and quantitated fluorometrically.⁵

Results.—*Demonstration of an antigen appearing in the blood during the incubation period of serum hepatitis:* Failure in the past to isolate a causative virus from serum hepatitis could possibly be attributed to the fact that most isolation attempts have been carried out with specimens obtained early in the *clinical* course of the disease, at what is actually a *late* stage of the infection due to the

long incubation periods characteristic of this disease. Infective viruses may be present only early in the disease, perhaps becoming masked by antibody when clinical symptoms become evident. We therefore attempted to obtain specimens from cases of serum hepatitis before exposure to the virus and at closely timed intervals thereafter throughout the entire course of the disease. As human volunteer experiments were not contemplated, it was necessary to follow a large number of transfused patients.

The first patient in this study (case MU-5) who developed a classical case of "long-incubation" serum hepatitis was chosen for detailed study. This patient was operated upon because of hemorrhage from a gastric ulcer. Sixteen transfusions were administered during the period of three to five months prior to onset of hepatitis. Following discharge from the hospital, the patient did well and was under no drug therapy. The possibility of drug-induced hepatitis thus appeared remote. Since the patient had no contact with children or known cases of jaundice, the probability of intercurrent exposure to infectious hepatitis virus was reduced. The results of clinical chemical tests carried out on the sera from this patient over a period of almost eight months are summarized in Figure 1. Approximately three months after surgery, a marked elevation of transaminase was observed, increasing progressively to a level of 2100 units. Concurrently, a parallel rise in LDH-5 was also observed. These findings suggested hepatocellular injury. A liver biopsy revealed focal necrosis, numerous eosinophilic bodies, variation in size and staining reaction of hepatocyte nuclei, prominent nucleoli, distortion of lobular architecture, and no evidence of fibrosis. All these features were compatible with the diagnosis of serum hepatitis, acquired most probably as a result of blood transfusion.

Serial twofold dilutions of each of the sera drawn from patient MU-5 were tested by double diffusion in agar against serum *S*. The results of these titrations are also shown in Figure 1. Concurrent with the first alteration of SGPT, a reactant that precipitated in the double diffusion technique with serum *S* appeared in the patient's serum. This reactive material, which I shall call SH, reached peak concentration approximately six weeks earlier than did the transaminase; it then rapidly declined in concentration and remained detectable for approximately six weeks longer.

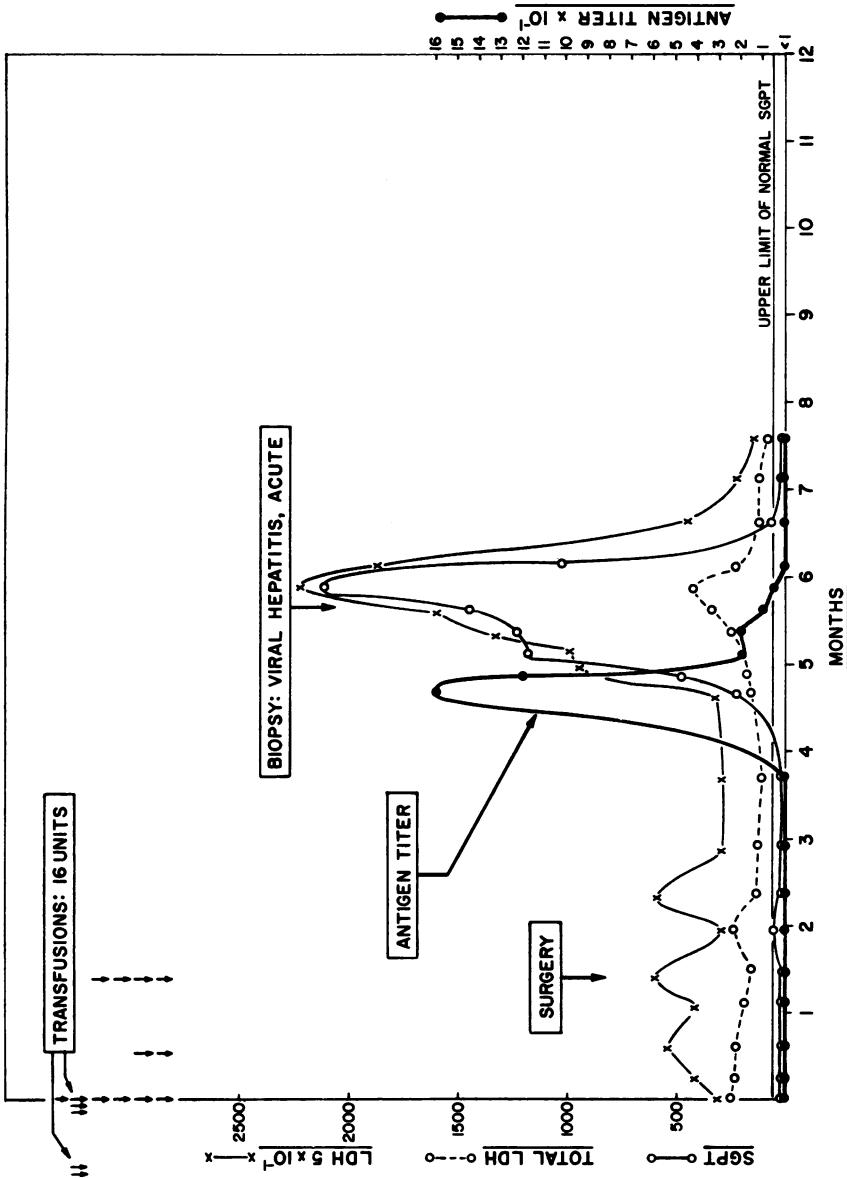
Since both reactants in the double diffusion reaction were contained in serum, it could not be determined *a priori* which contained antigen and which contained antibody; indeed, the precipitation reaction seen in these experiments might not be immunological at all. A number of experiments were therefore done to shed light on the mechanism of the phenomenon observed.

Nature of reactive component in serum S: To determine whether the precipitant was specific for serum *S* or could be found in other sera, the eleventh serum specimen from case MU-5, taken approximately when the reactive material reached peak concentration, was tested against serum *S*, as well as against five randomly chosen normal blood donor sera. The precipitation reaction occurred only with serum *S*.

Two experiments were carried out to determine whether or not the reactant in serum *S* was antibody. First, the serum was fractionated by ethanol fractiona-

Fig. 1.—Appearance of reactive material in serum during the incubation period of serum hepatitis.

Blood samples were obtained from patient MU-5 at approximately 2-week intervals and were assayed for SGPT, total LDH, and LDH isoenzymes. Remaining serum was held frozen. Serial 2-fold dilutions were subsequently assayed by double diffusion in agar for a precipitant reactive with undiluted serum S.



tion (see *Materials and Methods*). The titer of reactive material concentrated in fraction II was what would be expected if this material had been totally precipitated into this fraction. This experiment suggested that the precipitating agent was located in the γ -globulin fraction of serum *S*.

Additional evidence concerning the nature of the reactant in serum *S* was obtained by immunoelectrophoresis. It was found that the reactive material in serum *S* migrated towards the cathode at pH 8.6 at a rate indistinguishable from γ -G globulin. The reactant in serum *S* was therefore termed "antibody" and that in sera giving precipitation reactions with serum *S* was termed "antigen."

Relationship between SH antigen and serum hepatitis: In order to investigate further the possible relationship between SH antigen and viral hepatitis, it was necessary to study sera from various types of viral hepatitis, and it was thought desirable that these sera be studied in a double blind manner. For this purpose, the cooperation of Dr. Robert McCollum of the Department of Public Health, Yale University School of Medicine, was solicited. Dr. McCollum provided us with 60 coded sera from patients with serum hepatitis (four cases), unidentified or infectious hepatitis (six cases), infectious mononucleosis (two cases), and from nine prisoners with a history of drug addiction. The results of tests with these sera are summarized in Table 1. Five strong positive reactions were observed.

When these results were decoded, it was found that three of the positive sera came from one patient, who developed a classical case of serum hepatitis and whose history is summarized in footnote *d* of Table 1. In addition, a positive reaction was observed with the acute-phase serum specimen of another case of hepatitis, which was thought to have been due to accidental inoculation with infective serum derived from the first case. Two subsequent sera from the second case, drawn later in the course of the disease, were negative. A positive reaction was also detected in the acute-phase specimen, but not in convalescent-phase specimens, taken from a student who developed viral hepatitis after spending a summer in Europe.

None of the sera taken from cases of infectious hepatitis, infectious mononucleosis, and ex-addicts gave positive reactions. It was concluded that serum *S* appeared to react preferentially with material in *early* serum specimens from cases of serum hepatitis.

Further support for an association between SH antigen and serum hepatitis has been obtained in another double blind experiment carried out with Dr. Saul Krugman. Serial sera from four cases of long-incubation-period viral hepatitis produced by inoculation,⁶ as well as serial sera from four control patients, were tested under code for the presence of SH antigen. As shown in Table 2, SH antigen was detected in all four cases of long-incubation-period viral hepatitis and in none of the controls. In three patients, antigen was present only for a limited period, before or near the time when transaminase reached peak values. In one patient, SH antigen was found in the blood as late as 203 days after inoculation.

Distribution of antigens reacting with serum S: Using the double diffusion technique, we examined 2856 sera from normal blood donors and from some additional cases of serum hepatitis for precipitin reactions resulting from diffusion against serum *S*. Only 3 of the 2856 normal sera reacted positively. Eight

TABLE 1. Identification of three cases of viral hepatitis among 60 coded specimens.^a

Case	Diagnosis	Specimen	Date	Days since onset of symptoms	SGPT ^b (karmen U/ml)	Test for SH antigen (positives/number of tests done) ^c
K. B. ^d	Serum hepatitis	64-283	Dec. 18, 1964	8	41.0	10/10
		64-297	Dec. 31, 1964	20	42.6	6/7 ^e
		KB-1	Mar. 5, 1964	84	137.0	10/10
K. J. ^e	Serum hepatitis	65-229	Apr. 12, 1965	7	349	10/10
		h	Apr. 19, 1965	14	h	0/2
		h	May 25, 1965	51	h	0/2
W. S. ^f	Unidentified viral hepatitis	64-97	Oct. 1, 1964	9	100.3	5/6
		h	Oct. 8, 1964	16	h	0/2
		h	Oct. 22, 1964	30	h	0/2
		h	Nov. 3, 1964	41	h	0/2
		h	Apr. 22, 1965	210	h	0/2
Negative cases ^g	Infectious hepatitis (5 cases) Infectious mononucleosis (2 cases) Serum hepatitis (2 cases) Ex-addicts (9 cases)				Various	0/2

^a Specimens supplied by Dr. R. McCollum, Department of Epidemiology and Public Health, Yale University School of Medicine.

^b Tested at The New York Blood Center, January 1968. Specimens were stored at -20°C .

^c Tested by immunodiffusion technique against concentrated globulin fraction of serum S.

^d A patient who developed a classical case of serum hepatitis 49 days after transfusion of 9 units of blood, one of which had been drawn from a medical resident who subsequently developed a severe case of viral hepatitis.

^e A laboratory technician who carried out liver function tests and was thought to have acquired her infection by accidental inoculation with a specimen from case K. B. above.

^f A student who developed viral hepatitis following his return from a summer vacation in Europe. The nature of the exposure is not known.

^g Rare nonreproducibility may result from inadvertent misfilling of wells.

^h Because of other experiments in progress, only positive specimens had been decoded at the time this report was prepared.

of the ten sera from serum hepatitis cases taken during the first week of clinical illness contained detectable antigen. Such a finding supports the association between this antigen and serum hepatitis.

Relationship among SH antigens detected in different patients: Materials giving precipitin reactions with serum S were detected in 23 sera from 16 different patients. In five cases, reactive material was detected in more than one serum sample. The possibility existed that a variety of disparate antigens had been identified with S antiserum. Therefore, the positive specimens were tested against S antiserum for reactions of identity. Such reactions, in which adjacent precipitin lines fuse without crossing or "spurring" and in which inhibition can be demonstrated in the zone of fusion, are thought to indicate immunologic identity of antigens. A typical reaction of identity is illustrated in Figure 2. Identity tests carried out on 16 sera from 12 different cases showed reactions of identity among all 16 sera and thus provided evidence for the existence of the same antigen (SH) in these different patients. The antigen detected in the Willowbrook cases (Table 2) also showed reactions of identity with reference SH antigen.

Distribution of antibody to SH antigen: Sera were next examined for the pres-

TABLE 2. *Identification of four cases of long-incubation-period viral hepatitis (Willowbrook School).^a*

Case	Age and Sex	Serum no.	Inoculum	Days after inoculation	SGOT ^b (Reitman-Frankel U/ml)	Bilirubin	Test for SH antigen ^c
N. C.	9-year-old male	K-2	MS-2 i.m. ^e	6	12	<1	0 ^d
		20-68		48	89	<1	+
		—		55	680	<1	—
		K-9		113	78	<1	0
		K-25		203	8	<1	0
B. L.	9-year-old male	K-10	MS-2 i.m.	6	20	<1	0
		39-68		66	84	2.62	+
		—		90	1380	1.82	—
		K-28		104	94	<1	+
		K-3		163	22	<1	0
J. R.	5-year-old male	K-17	MS-2 i.m.	6	21	<1	0
		40-68		66	150	<1	+
		—		76	620	<1	—
		—		104	620	<1	—
		12-68		171	106	<1	+
		11-68		203	34	<1	+
J. C.	3-year-old female	K-8	Uninoculated	6	36	<1	0
		K-27		55	64	<1	0
		K-1		155	144	<1	0
		—		215	640	1.4	—
		17-68		235	50	<1	+
		K-18		308	64	<1	0
Controls		14 sera from 4 normal children					0

^a Specimens supplied by Dr. S. Krugman, New York University School of Medicine.

^b Test carried out on fresh serum specimens.

^c Test in duplicate by immunodiffusion technique against 5 × concentrated globulin fraction of serum S.

^d (0) no antigen detected; (+) antigen detected in duplicate tests; (—) not tested.

^e Krugman's MS-2 strain (long-incubation-period) of hepatitis virus inoculated intramuscularly.⁶

ence of *antibody* detectable by the double diffusion test, with the eleventh serum from case MU-5 (Fig. 1) as antigen. Antibody was not found in five sera from convalescent serum hepatitis patients. Antibody was, however, found in 2 of 24 multiply transfused Cooley's anemia patients and in 1 of 12 multiply transfused hemophilia patients tested.

Discussion.—An antigen that appeared during the course of the incubation period of a classical case of long-incubation-period, post-transfusion viral hepatitis has been found with the immunodiffusion technique. The data presented indicate that detectable antigen is associated with serum hepatitis and that it is extremely rare in the normal population.

It should be emphasized that the prevalence of the antigen and antibody observed in this study must be taken to refer only to prevalence of quantities detectable with the techniques employed. The insensitivity of the immunodiffusion technique offers certain advantages with regard to specificity, but probably reveals only a small fraction of the total picture. It is likely that a more

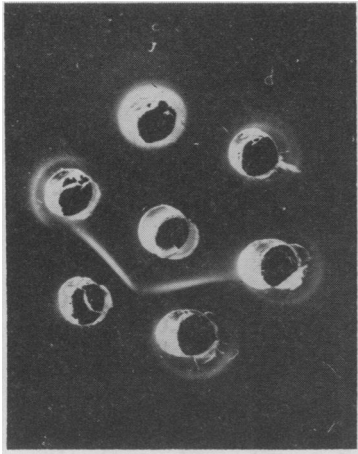


FIG. 2.—Reaction of identity. The center well contained $5\times$ concentrated *S* antiserum. Peripheral wells contained a variety of control sera and sera from two patients with mongolism (5-67 and 6-67). A typical reaction of identity is evident.

sensitive test would detect antigen and antibody in a higher proportion of the sera tested.

At least three hypotheses can be entertained to account for the phenomenon of the SH antigen: first, the possibility that this antigen is located on a virus particle and that this virus particle is etiologically related to some or all cases of serum hepatitis; second, the possibility that the SH antigen is a nonvirion-associated viral antigen, i.e., a soluble viral antigen; and last, the possibility that the SH antigen represents a product of the host and that its appearance during the incubation period of the disease is perhaps related to the results of virus infection.

The data presently available favor the first of the above hypotheses, although the first and last hypotheses are not mutually exclusive. The results obtained in the coded experiments suggest that SH antigen is not a nonspecific product of liver injury. This antigen was not seen in patients with marked transaminase

elevations resulting from infection with the viruses of infectious mononucleosis or infectious hepatitis (virus A). Preliminary results from studies now under way suggest that the SH antigen is located on a viruslike particle having an electron microscopic morphology, chemical composition, and density similar to those of arboviruses, and a diameter of approximately $25\text{ m}\mu$. These results will be reported in detail separately.

It thus appears likely that measurement of SH antigen constitutes detection of serum hepatitis virions. This serves to explain the resemblance of the kinetics of the appearance and disappearance of this antigen to that of a classical viremia curve. The apparent persistence of SH antigen 203 days following its appearance in case J. R. (Table 2) is thus suggestive of the induction of a "carrier state" in this patient.

Summary.—An antigen, termed SH, has been identified in serum during the incubation period of a classical case of post-transfusion serum hepatitis. It has also been found in 7 out of 9 (6 of the latter under code) additional cases of serum hepatitis and in 3 out of 2856 normal blood donors tested.

Antibody to SH antigen was found in patients who had been multiply transfused, such as patients with hemophilia and Cooley's anemia. The antibody was not detected in sera from convalescent patients with typical cases of viral hepatitis.

SH antigens found in the sera of 16 separate individuals all showed reactions of identity in immunodiffusion tests.

Note added in proof: Since the completion of this manuscript, additional coded experiments have been carried out. In collaboration with Drs. W. P. Parks, W. R. Rawls, and J. L. Melnick, Baylor University College of Medicine, SH antigen was detected in acute sera from two cases of serum hepatitis and from one case of viral hepatitis of unspecified etiology. Antigen was not detected in two other cases of hepatitis. In addition, attempts to demonstrate SH antigen in acute and convalescent sera from marmosets inoculated with the Barker strain of marmoset hepatitis virus were unsuccessful.

In a second coded experiment carried out with the help of Dr. Saul Krugman, SH antigen was detected in a MS-2 serum pool taken from patients inoculated with this strain of virus, and in an additional serum specimen taken from case J. R. (Table 2) 442 days after inoculation. In this latter case, the detection of antigen more than one year after inoculation suggests that a carrier state has been induced in this patient. Antigen was not found in serial sera from two cases inoculated with MS-1 (short-incubation-period) Willowbrook strain of hepatitis virus.

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