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Hepatitis-Associated Antigen

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Identification of an antigen that is intimately related to the causative agent of viral hepatitis has given impetus to hepatitis research. Blumberg and associates [1] found this antigen incidentally while working on precipitins of β-lipoprotein allotypes. The precipitin was in the serum of a patient with hemophilia who had received multiple transfusions, and the antigen happened to be in serum from an Australian aborigine. The precipitin line that formed in Ouchterlony double diffusion differed in specificity from known β-lipoproteins, and the antigen was called Australia antigen or Au(1). The antigen was considered to be inherited as a recessive trait based on statistical analyses of its occurrence in large tropical populations [2]. A relationship was gradually recognized over the next four years between Australia antigen and leukemia, Down's syndrome, lepromatous leprosy and hepatitis, and the possibility was considered that the Au(1) trait might be associated with susceptibility to viruses [3,4]. Okochi and Murakami [5] confirmed the association between Australia antigen and hepatitis and demonstrated the appearance of antigen during the incubation period and acute phase of classic post-transfusion viral hepatitis. Prince [6] reported similar observations on patients with serum hepatitis and called the antigen, SH. Examination of an immune precipitate of Au(1) by electron microscopy showed aggregates of 200 Å particles [7], and shortly thereafter the close relationship between Australia antigen, the occurrence of viral hepatitis and the presence of virus-like particles in antigenic serum was confirmed [6,8–11]. Although several different names have been applied to the antigen, only a single well documented specificity exists, and many workers have adopted the more general term, hepatitis-associated antigen (HAA), rather than the genetic nomenclature, Au(1), of Blumberg et al., or the more circumscript SH antigen of Prince.

This review summarizes information on the physical and chemical characteristics of the hepatitis-associated antigen (HAA), the clinical circumstances under which HAA and anti-HAA occur, the association between HAA and transmission of hepatitis, the relationship between anti-HAA and immunity, and the suitability of various technics for measuring HAA and anti-HAA.

PHYSICAL AND CHEMICAL CHARACTERISTICS OF HAA

All information to date on HAA suggests that it is chiefly the protein coat or capsid material of a small virus. There appears to be some associated infectious virion, but it is too sparse to characterize.

Protein-Like Characteristics. In 1966, Alter and Blumberg [12] found that HAA was a macromolecule which appeared in the

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first peak of serum filtrates on Sephadex® 200 gel, traveled as an α globulin in gel electrophoresis, appeared to contain a small amount of lipid on the basis of a weak reaction with Sudan black, and differed from other lipoproteins in its immunoreactions and density. HAA was more dense than β-lipoproteins, being in fractions that sedimented in potassium bromide at specific gravities between 1.063 and 1.3, and lighter than most serum proteins which were in fractions of specific gravity >1.3. The antigen was considered to be possibly an altered, partially delipidized lipoprotein until its virus-like nature was revealed by electron microscopy of precipitates of HAA formed with specific antiserum [7]. These precipitates contained conglomerates of particles approximately 200 Å in diameter with some elongated structures of the same diameter varying in length up to 2,300 Å. Within a year several groups of investigators reported the presence of the same virus-like particles in serum that was positive for HAA by immunologic tests [8–11].

Virus-Like Characteristics. HAA particles aggregated by antibody can be pelleted in ten minutes at 25,000 g and are readily seen after negative staining (Figure 1). The particles have a modal diameter of approximately 200 Å (Figure 2A); in some preparations, an occasional particle appears to have a central core

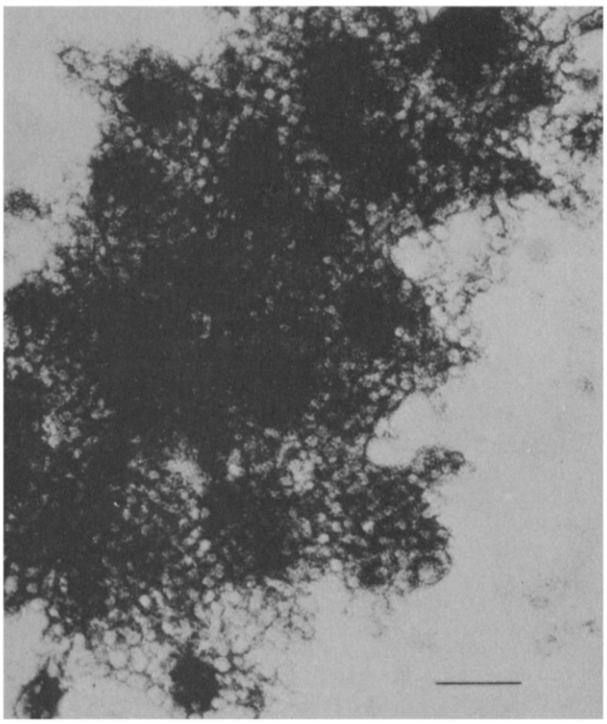

Figure 1. Electron micrograph of HAA particles aggregated by anti-HAA, pelleted, and negatively stained with uranyl acetate. Bar at the lower right equals 1,000 Å. From Hirschman et al. (JAMA 208: 1667, 1969) [8].

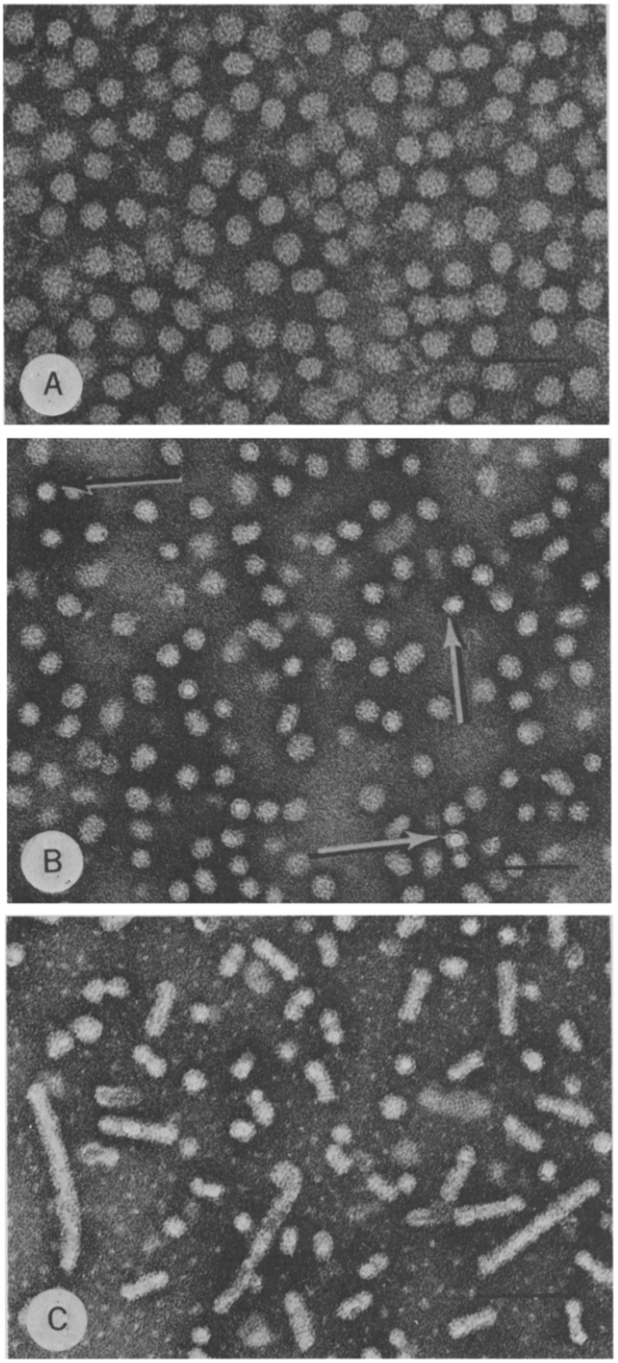

Figure 2. Electron micrographs of HAA particles. A, characteristic appearance of HAA particles concentrated by isopycnic banding on cesium chloride. The particles appear relatively homogeneous with a modal diameter of approximately 200 Å. Particles negatively stained with phosphotungstic acid. Bar equals 500 Å. B, occasional HAA particles from some serum specimens appear to have dense cores, indicated by arrows. Bar equals 1,000 Å. C, filamentous forms of HAA particles concentrated by differential rate sedimentation in cesium chloride gradients. Bar equals 1,000 Å. These electron micrographs were taken by Dr. William Hall [13].

(Figure 2B); and usually a few conspicuously large spherical particles are seen (Figure 2C). Different serum specimens contain varying numbers of tubular or filamentous forms which show periodicity (Figure 2C). Because of their size, filamentous and large spherical forms can be separated from the more uniform 200 Å spherical forms by differential rate sedimentation in cesium chloride gradients [13]. Organic solvents disrupt tubular forms, suggesting that they may consist of spherical particles inside a common lipid-containing membrane [9], perhaps of cellular origin.

Ether treatment reduces the diameter of the spherical particles by approximately 40 Å (Figure 3), suggesting removal of a 20 Å outer coat. The coat may contain some lipid, for the density of the particles measured in cesium chloride gradients increases from approximately 1.23 before treatment to approximately 1.28 after treatment with organic solvents [9].

One report [11] suggests that treatment with mild detergents such as Tween® 80, or freezing and thawing, may remove lighter capsid material and increase the density of HAA to the range of 1.35 to 1.4 on cesium chloride gradients; the apparently heavier HAA could be detected only by complement fixation and not by electron microscopy. Some investigators think that particles with apparent "core" material or "inner bodies" represent complete virus or infectious virion, but these particles have not been separated as yet from the more numerous apparently "empty" particles to determine whether they contain nucleotides.

Dane et al. [14] have reported 420 Å particles, similar to the larger spherical particles shown in Figure 2C, which they considered to be the complete virus in HAA-positive specimens from three patients. These particles probably were not aggregates of smaller spheres, for they were not disrupted by ether [14] as are tubular forms [9]. Although the 420 Å particles appeared to be slightly more dense than the 200 Å particles in cesium chloride gradients, the two sizes could not be separated by isopycnic banding.

Millman et al. [15], have reported that tests for nucleotides were negative on purified HAA used in sufficient amount to permit measurement of ribonucleic acid (RNA) if it were 10 per cent, or deoxyribonucleic acid (DNA) if it were 1 per cent of the total weight of protein in the preparation. Similar attempts by other investigators to measure RNA or DNA in purified HAA have given negative results. The low density of most HAA preparations therefore appears to be due to lack of nucleotides in the particles as well as to presence of some lipid.

The size of the HAA particle is the same as that of the small viruses in the picornavirus, parvovirus, arborvirus or enterovirus groups, but precise classification has not been possible because the attributes of the particles are not characteristic of any one group. The angularity of the outline of some particles and the apparent surface structures of approximately 30 Å subunits with no uniform arrangement (Figure 3), resemble features of defective adeno-associated virus [9,16]. However, the picornaviruses, parvoviruses and adeno-associated virus contain no lipid and have a higher buoyant density of 1.38 to 1.41 in cesium chloride, reflecting a relatively high nucleotide content [17,18]. HAA resembles some arborviruses in size, lipid content, and density; and resembles some enteroviruses in size and stability. However, HAA differs serologically from the known arborviruses, enteroviruses and other viruses, for complement fixation reagents representing 300 serotypes of picorna-, parvo- and arborviruses failed to fix complement with HAA or anti-HAA [19].

The particles are very stable. Morphologic characteristics of HAA and its antigenicity by immunodiffusion and complement fixation remain unchanged after heating at 60°C for one hour, after storage at room temperature for at least six months, or at —20°C for more than twenty years [8,9]. After treatment with organic solvents minor changes, such as shown in Figure 3, are evident, but antigenicity may actually be increased, perhaps because new sites are uncovered by removal of lipid or associated antibody [20].

Correlation with Observations on Infectivity. McCollum's filtration experiments in 1952 [21] to determine the size of the serum hepatitis agent as judged by infectivity in human beings showed that the serum hepatitis agent could pass through a filter of 520 Å average pore diameter. Since the particle would have to be approximately half the diameter of the filter pore, or <260 Å, to permit its passage, the infectious agent is probably the size of the most abundant HAA particles.

By titering infectious plasma in experimental subjects, Murray [22] estimated the concentration of infectious particles in serum from patients with serum

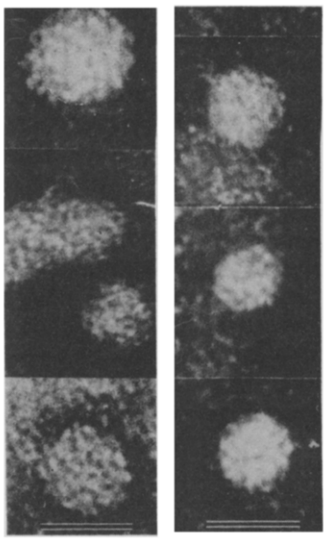

Figure 3. HAA particles separated by isopycnic banding on cesium chloride gradient, negatively stained. Bars at bottom equal 200 Å. Particles on left untreated; particles on right after ether treatment. Note the apparent surface subunits of approximately 30 Å. From Parker et al. (J Immun 102: 1529, 1969) [9].

TABLE I Characteristics of HAA

Data	Source	Characteristics
Morphology	[7–11]	Spherical (possibly icosahedral), approximately 200 Å in diameter; subunits on surface 30 Å not uniform; some filamentous forms 200 Å up to 2,300 Å; outer coat 20 Å removal with lipid solvents; occasional 420 Å spherical particles; central core apparent rarely
Density	[7,9,11,21]	1.20–1.24 on cesium chloride gradients, 1.26–1.29 after ether extraction; similar increase after exposure to sodium deoxycholate; 1.39–1.41 possible small fraction after exposure to Tween 80
Nucleotide content	[16]	No DNA or RNA detected as yet
Stability	[9,11,12,14,20]	Morphologically and immunologically stable after exposure to 60° > 1 hr, 27° > 6 mo, −20° > 20 yr, pH 2.7 > 3 hr; and to ether, ethanol, chloroform, genetron 113 all > 2 hr at 27°; morphologically and immunologically unstable after exposure to sodium dodecyl sulfate or ethanol:glacial acetic acid (3:1); immunologically unstable but morphologically stable after exposure to PO_4 buffers, 85°–100°C > 15 min, ammonium sulfate precipitation, sedimentation on sucrose gradients, and freezing when purified
Protein-like characteristics	[12]	Elutes from DEAE with β-lipoprotein, IgM, IgA, albumin, ceruloplasmin, α_2 macroglobulins; moves in agar gel electrophoresis like α globulin; stains weakly with lipid stains (e.g., Sudan black), strongly with protein stains (e.g., azocarmine); separates in first peak of serum filtrates on Sephadex 200 gel
Distribution of HAA in Cohn ethanol fractionations of highly contaminated plasma	[24]	Fraction I (fibrinogen, factor VIII) low concentration; fraction II (normal immunoglobulin) none; factor IX complex (also II, VII, X) low concentration; subfraction III (thrombin) high concentration; fraction V (albumin) none or low

NOTE: Information not in references are my personal observations.

TABLE II Incidence of HAA in Patients with Acute Hepatitis

		Type of Hepatitis					
		Infectious		Serum		Serum or Infectious	
Source	Technic	No.	% HAA+	No.	% HAA+	No.	% HAA+
Blumberg et al. [25]	Gel diffusion	84	13	41	34	...	...
Okochi, Murakami [5]	Gel diffusion	66	15	101	13	...	...
Gocke, Kavey [26]	Gel diffusion	15	47	48	75	14	43
Hirschman et al. [8]	Gel diffusion	151	14	62	74	...	...
Wright et al. [27]	Gel diffusion	12	25	43	49	33	67
Cossart, Vahrman [28]	Gel diffusion	51	47	10	50	7	60
Shulman et al. [29]	Comp. fixation	151	33	130	93	...	...
Giles et al. [30]	Comp. fixation	31	0	19	95	...	...
		"MS-1"		"MS-2"			
Total		379	27	383	67	54	59

NOTE: The clinical differentiation of infectious and serum hepatitis in these cases (except for those caused by MS-1 and MS-2) was based chiefly on incubation period after the implicated parenteral exposure, short-incubation (two to six weeks) being called infectious hepatitis, long-incubation (six to twenty-six weeks) serum hepatitis. Most cases in which there was no parenteral exposure were considered to be infectious hepatitis, and some were not differentiated. The MS-1 and MS-2 cases were all produced by injecting material known to produce short- and long-incubation-period hepatitis, respectively. MS-1 and MS-2 cases were excluded from the totals, as were the cases of Hirschman et al. which were subsequently analyzed with the more sensitive complement fixation technic (Shulman et al.).

hepatitis in the acute phase (acute phase serum) to be 10^6/ml [22]. The concentration of HAA particles counted by electron microscopy in similar acute phase serum is usually greater than 10^{10}/ml. In view of this disparity and biologic evidence (presented later) for transmission of hepatitis by materials containing HAA, it seems likely that material identified as HAA is chiefly "noninfectious" empty virus coat. Although incomplete virus predominates some complete infectious virus is present.

This observation on the physical and chemical stability of HAA are consistent with the known stability of the infectious agent of hepatitis, for contaminated plasma transmits the disease after similar treatment with heat or solvents, and after similar periods of storage [23].

Physical and chemical attributes of HAA, including the distribution of HAA in Cohn ethanol fractions of human plasma [24], are summarized in Table I.

RELATIONSHIP BETWEEN HAA AND DISEASE

HAA in Acute Hepatitis. Frequency of HAA in acute hepatitis: After the initial suggestion in 1967 by Blumberg and co-workers [4] that Australia antigen might be related to the infectious agent of viral hepatitis, and the demonstration in 1968 by Okochi and Murakami [5] and Prince [6] that the antigen appeared in blood of patients during the incubation period of serum hepatitis, a number of investigators reported further evidence for existence of a hepatitis-associated antigen (Table II). The over-all frequency of HAA in the 866 cases of acute viral hepatitis was 45 per cent.

In control populations (Table III) only 0.1 to 0.2 per cent of hospital personnel and patients without liver disease had HAA in their blood; the same low frequency was seen in patients with liver disease or hepatitis clearly unrelated to viral hepatitis as in patients without liver disease. The frequency of HAA in blood donors in New York was 0.73 per cent; and in different groups of blood donors in Tokyo, 0.19 to 1.23 per cent (Table III).

Patients with viral hepatitis show a variable frequency of positive tests for HAA depending not only on differences in the number of samples taken per patient and on the stage of the disease at which serum is tested, but also on the technic used for measuring HAA. The highest frequencies are found when sampling is early in the course of the disease rather than late in the active phase or in the convalescent phase [8,26,28–30], when more than one sample is taken at about weekly intervals [8,28,29], and when a more sensitive technic such as complement fixation (CF) is used rather than the immunodiffusion technic [28–30].

Identity between "HAA-positive" and "long-incubation" hepatitis: Generally the frequency of HAA was greater in patients with documented post-transfusion or post-injection hepatitis than in patients with no history of parenteral exposure. In those series in which all patients were tested during the early acute phase of the disease, the frequency of HAA varied from 50 to 95 per cent for hepatitis acquired parenterally and from 25 to 47 per cent for sporadic cases of hepatitis that were considered to be infectious (Table II). None of the patients listed in Table II were involved in a so-called "single source" outbreak of short-incubation infectious hepatitis, and well documented epidemics of short-incubation viral hepatitis involving person to person spread have not as yet been associated with positive tests for HAA [33]. Diagnosis of serum hepatitis can be established with certainty by the incubation period after parenteral exposure, but if there is no reasonable history of parenteral exposure the diagnosis is usually considered to be infectious hepatitis even if the source of infection and the incubation period are uncertain. Therefore, in Table II both long-incubation and short-incubation viral hepatitis cases are no doubt included in the groups of patients with the diagnosis of infectious hepatitis. In view of the following evidence that the long-incubation type of viral hepatitis is associated with HAA and the short-incubation type is not, and that both types can be transmitted parenterally or orally, it is possible that all the HAA-positive cases listed in Table II in which the diagnosis was infectious hepatitis were long-incubation hepatitis.

Early work on the oral and nasopharyngeal transmission of long-incubation hepatitis was equivocal or negative [35,36]. However, there is a noteworthy epidemiologic study indicating that this form of hepatitis can be spread by intimate contact [37]; there are recent reports on epidemics of long-incubation hepatitis in patients and staff on renal dialysis units sug-

TABLE III Frequency of HAA in Control Populations

Source	Group	No.	% HAA+
Blumberg et al. [25]	Nonhospitalized, healthy	2,412	0.1
	Hospital patients without liver disease	1,055	0.2
	Laennec's cirrhosis	52	0
Gocke et al. [31]	N. Y. blood donors	2,211	0.73
Okochi, Murakami [5]	Tokyo blood donors		
	Male	4,222	1.23
	Female	1,024	0.19
Wright et al. [27]	Neoplasm	11	0
	Drug hepatitis	23	0
	Laennec's cirrhosis	57	3.5*
Gocke, Kavey [26]	Cirrhosis, biliary obstruction, drug hepatitis, cancer	36	0.3
Fox et al. [32]	Alcoholic liver	31	0

* HAA+ patients were given transfusions.

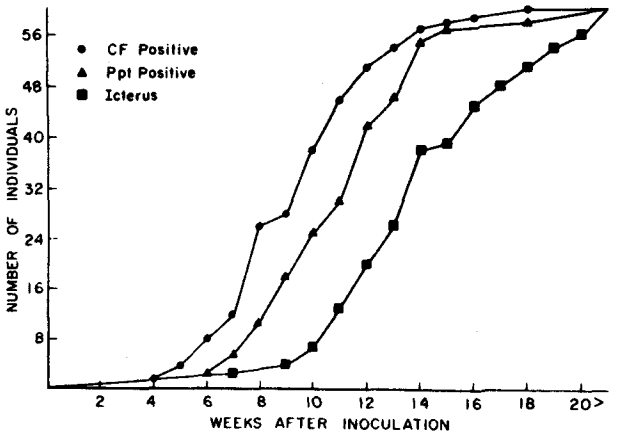

Figure 4. Cumulative frequency with time of icterus in sixty cases of serum hepatitis compared to appearance of HAA in serum detected by complement fixation and agar gel precipitation. From Shulman et al. (Ann Intern Med 72: 257, 1970) [29].

gesting person to person spread [38,39]; and epidemiologic and experimental observations by Krugman and co-workers [40] indicating that institutional, endemic, long-incubation hepatitis can be transmitted nonparenterally. The studies of Krugman et al. clearly demonstrate two distinct hepatitis agents, one responsible for long- and the other for short-incubation hepatitis. The short-incubation agent, called MS-1, produces hepatitis thirty-five to forty-seven days after injecting or feeding infectious serum or stool filtrates, whereas the long-incubation agent, called MS-2, produces hepatitis forty-one to 108 days after injection and approximately seventy to 130 days after feeding infectious serum. It is noteworthy that the oral dose necessary to produce the delayed long-incubation hepatitis was fiftyfold higher than the parenteral dose, indicating that infectivity is markedly decreased when the agent is transmitted orally.

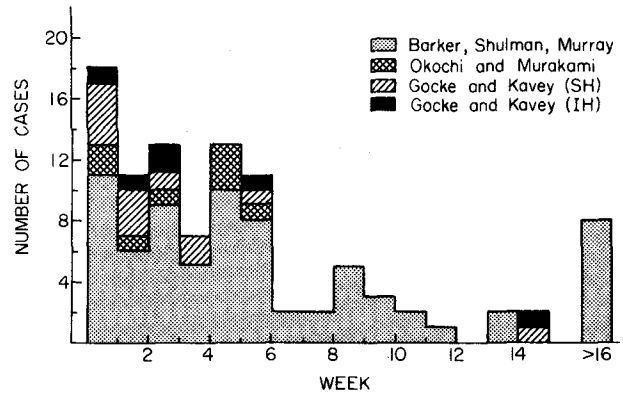

Figure 5. Duration of HAA in the sixty patients shown in Figure 4 and in the patients of Okochi and Murakami [5] and of Gocke and Kavey [26]. Duration of HAA in Gocke and Kavey series estimated from data given. HAA in each series measured by agar gel diffusion.

Giles and co-workers [30] found that almost all patients given the long-incubation MS-2 agent have HAA in their serum during the incubation period or acute stage of hepatitis, whereas patients given the short-incubation MS-1 agent never have HAA in their serum (Table II). In our experience, HAA found in patients diagnosed as having "serum" or "infectious" hepatitis has identical immunologic specificity [8,29]. Short incubation (infectious) hepatitis and long-incubation (serum) hepatitis are known to confer homologous but not heterologous immunity [35,36] as do the MS-1 and MS-2 agents of Krugman, et al. [34,40]. Thus, at least two viral agents cause the same clinical manifestations of hepatitis and can be transmitted in the same ways, but one has a long-incubation period and produces HAA, whereas the other has a short incubation period and has not yet been defined immunologically.

Appearance and persistence of HAA: The temporal relationships between exposure to hepatitis, appearance of HAA in blood, and development of symptoms and abnormal liver function tests were well documented in studies involving serum specimens collected from sixty subjects who had been inoculated with icterogenic plasma in hepatitis transmission experiments carried out between 1951 and 1954 [8,22,29]. Figure 4 is a cumulative plot with time of subjects in whom jaundice developed and in whom the complement fixation and immunodiffusion tests for HAA became positive. Figure 5 shows the duration of HAA in these same patients and in additional patients studied by other investigators [5,26].

With the more sensitive complement fixation technic, HAA appeared, on the average, four weeks before clinical or laboratory evidence of liver dysfunction, although this interval varied in individual cases from one day to seven weeks. Krugman and Giles [34], using complement fixation technics, found that HAA appeared two weeks to two months before abnormal levels of serum glutamic oxalic transaminase (SGOT) in patients inoculated with the MS-2 hepatitis agent. The less sensitive agar gel diffusion technic detected HAA about two weeks after the complement fixation technic (Figure 4) at about the same time that elevation of serum glutamic pyruvic transaminase (SGPT) could be measured [5,6]. HAA that appeared during the incubation period of experimental hepatitis persisted in almost all cases until onset of symptoms and abnormal liver function tests [29,34]. In most cases of naturally occurring hepatitis, serum obtained in the first week of the acute phase was most likely to be positive for HAA [5,6,26], although among patients studied by Cossart and Vahrman [28] antigen developed in more patients during the second week of illness.

Persistence of antigen in blood (Figure 5) has been

found by a number of investigators to range from a few days to many years [5,6,8,25,26,28–30]. In most cases HAA disappeared before symptoms subsided or biochemical abnormalities returned to normal, although in some cases the reverse was true. There was no significant difference in duration of HAA in the eighty patients diagnosed as having "serum" hepatitis in (Figure 5), the six patients diagnosed by Gocke and Kavey [26] as having "infectious" hepatitis, in the patients diagnosed by Cossart and Vahrman [28] as having "serum or infectious" hepatitis, or in subjects inoculated with the MS-2 agent [34]. In all the various studies there appeared to be no consistent correlation between persistence of antigen up to sixteen weeks and time at which HAA was first detected in the blood, titer of HAA or severity of hepatitis [26, 28,29,34].

The "Carrier" State. Of eighty-six HAA-positive patients in experimental hepatitis transmission studies (Figure 5), eight had antigen in their blood at sixteen weeks, and it was still present in serum samples of all eight taken three years later. In the studies of Krugman and Giles [34], 35 per cent of institutionalized children who had a positive test for HAA after inoculation with the MS-2 agent retained the antigen in their serum for many months or many years, and the frequency of HAA persisting more than twenty-four months in asymptomatic adult patients in the same institution was approximately 18 per cent. Ordinarily, when HAA could be detected in a patient at four months after the onset of acute hepatitis, it was present indefinitely [29,34]. Those who have been observed to retain HAA recovered from the acute illness in a routine fashion and had either very mild or no laboratory or clinical evidence of hepatitis at the time HAA was still present four months to many years later. Although persistence of antigen may occur after hepatitis of all degrees of severity, it appears to occur more commonly after very mild or subclinical hepatitis than after overt clinical disease [29,34,38, 39,41,42]. Hence many carriers of HAA may be unaware of an antecedent episode of acute hepatitis. This is in keeping with recent observations that asymptomatic subjects who have HAA in their blood will also have histologic evidence of chronic liver disease [27,43,44] even when their liver function tests, including transaminase values, are normal and they have no history of antecedent hepatitis. The frequency of subclinical chronic hepatitis as judged by the incidence of HAA in apparently healthy populations would be about 0.1 per cent in the United States, 2 to 5 per cent in many tropical regions and as high as 20 per cent in certain endemic areas [2]. Evidence suggests that subclinical chronic hepatitis occurs more frequently the lower the dose of infectious virus (see section "Relationship Between Viral Dose, Mode of Transmission and Severity of Hepatitis").

HAA in Symptomatic Chronic Liver Disease. Progressive changes in serial liver biopsy specimens from acute viral hepatitis to cirrhosis have been well documented [27,45], and recently Sherlock and coworkers [46] observed these changes over a ten month period during which tests for HAA remained positive. Since HAA-positive acute viral hepatitis can be followed by a spectrum of chronic liver lesions, it is not surprising that a high frequency of HAA has been associated with certain categories of chronic liver disease (Table IV).

TABLE IV Frequency of HAA in Chronic Liver Disease

Source	Type of Liver Disease	No.	% HAA+
Wright et al. [27] New Haven, Conn.	Prolonged viral hepatitis:		
	Unresolved classic	14	50
	Subacute hepatic necrosis	15	27
	Chronic active hepatitis	24	25
	Inactive "cryptogenic" postnecrotic cirrhosis	26	4
	Primary biliary cirrhosis	44	0
Gitnick et al. [47] Rochester, Minn.	Chronic active hepatitis	31	10
Bulkley et al. [48] Boston, Mass.	Chronic active hepatitis	30	23
Mathews, Mackay [49] Melbourne, Australia	Active chronic hepatitis	53	4
Becker et al. [50] London, England	Chronic persistent hepatitis	14	0
Fox et al. [32] London, England	Active chronic hepatitis	32	0
	Primary biliary cirrhosis	39	0
	Prolonged viral hepatitis (>6 weeks)	7	40
	Chronic persistent hepatitis (>1 yr.)	19	0
	Primary liver cell carcinoma	33	6
	Cryptogenic cirrhosis	49	2
Prince, Burke [51] New York, N. Y.	Primary biliary cirrhosis	10	90*

* This result was obtained with a so-called high voltage "immunoelectroosmophoresis" which is similar to an electrophoretic technic of Pesendorfer et al. [52]. Prince and Burke found no positive reactions with the gel diffusion assay used by Wright et al. and Fox et al., and also found no positive reactions with the technic of Pesendorfer et al.

Prolonged viral hepatitis: There is a high frequency of HAA in prolonged viral hepatitis whether the lesion is that of unresolved classic hepatitis or subacute hepatic necrosis (Table IV). The difference between these patients and asymptomatic carriers of HAA appears to be one of degree of hepatic involvement. The level of HAA does not seem to vary with fluctuations in liver function tests and is not related to the degree of abnormality of these tests or severity of the symptoms. HAA occasionally has been observed

to disappear in the face of persistent symptoms and abnormal laboratory tests but tends to persist even when activity of the disease is suppressed by adrenal corticosteroid therapy [27]. In all instances in which HAA was present in the blood of subjects who had liver biopsies, histologic lesions of chronic hepatitis were found even during remission when there were no symptoms and no abnormalities in liver function tests [27,43,44].

Patients in London with chronic persistent hepatitis have not had HAA in their blood [32,50]. However, all but one of the patients studied in one series had "infectious" hepatitis rather than "serum" hepatitis as the initial illness [50], and a high proportion of patients in the other London series probably had "infectious" hepatitis [32], whereas there was epidemiologic evidence of "serum" hepatitis in every case of prolonged hepatitis studied by Wright et al. [27] in the United States.

Chronic active hepatitis: Although some cases of chronic active hepatitis (CAH) (or active chronic hepatitis) appear to result from unresolved viral hepatitis, CAH seems to be a syndrome rather than a single disease [53], for many cases in this category appear to be closely related to lupus-like disorders. In several studies in the United States, 10 to 25 per cent of patients with CAH have had positive tests for HAA [27, 48–50]. The titers of HAA in patients with CAH are the same as in patients with acute viral hepatitis, i.e., usually 1:100 or greater by complement fixation; but in contrast to the transient nature of HAA in acute hepatitis, the titer tends to remain constant for many months to several years in CAH despite fluctuations in liver function tests and in face of various forms of treatment including adrenal corticosteroids [27,48]. However, HAA disappeared in several patients with CAH over a period of many months while clinical improvement occurred [27,48].

Although patients with CAH are predominantly female, those with CAH who have circulating HAA are predominantly male [48] and usually have had an antecedent episode of acute hepatitis with a strong history of parenteral exposure. Although about 15 per cent of patients with CAH have a positive lupus erythematosus cell phenomenon [53], a much lower per cent of patients with CAH and HAA have had a positive lupus erythematosus cell test [27,47,48], and almost all reported patients with CAH and a positive lupus erythematosus cell test, have not had a positive test for HAA. Thus the test for HAA seems to differentiate two groups of patients in the category of CAH: the HAA-positive group that most likely progressed from acute viral hepatitis, and the lupus erythematosus-positive, HAA-negative group that probably represents an immunologic aberration with lupus-like features. Subdivision of CAH cases in this way may prove to be of prognostic and therapeutic importance, particularly with respect to the use of adrenocorticosteroids.

Similar cases of CAH in England and Australia were not associated with positive tests for HAA [32,49]. Since levels of HAA in positive cases in the United States were usually high and easy to detect even with weak antibodies, technical differences do not seem to account for this discrepancy. Perhaps CAH in the United States is more often a sequela of HAA-positive viral hepatitis than it is in other countries in which this type of hepatitis is not as prevalent.

Cirrhosis: Cryptogenic cirrhosis or inactive postnecrotic cirrhosis, which in some instances may represent the inactive phase of CAH or the healed stage of acute viral hepatitis [53], has a low incidence of HAA (Table IV). Although it is considered that acute viral hepatitis may be the etiology of some cases of primary biliary cirrhosis, the disease is most likely not related to the HAA-positive form of hepatitis, for of eighty-three cases studied by two groups of investigators all were negative for HAA (Table IV). Laennec's (alcoholic) cirrhosis is not associated with HAA unless the patients have received transfusions (Table III).

Recently Prince and Burke [51] reported positive tests for HAA in nine of ten patients with primary biliary cirrhosis, using a high voltage counterelectrophoresis technic, but found no positive reaction with a lower voltage technic [52]. We have found that the high voltage and low voltage technics are identical in sensitivity, but the high voltage technic according to Prince and Burke is subject to false positive reactions. Perhaps this is the basis for the apparent increased sensitivity of the high voltage over the low voltage technic and also for the discrepancy between their results in biliary cirrhosis and those of others.

Conditions Possibly Predisposing to HAA-Positive Hepatitis. A number of reports by Blumberg and coworkers suggest that patients with Down's syndrome, lepromatous leprosy, leukemia or uremia have a high frequency of HAA because they are unusually susceptible to hepatitis by virtue of a genetic or acquired immunologic defect [3,4,25,54,55]. Moreover, on the basis of extensive family studies, Blumberg et al. concluded that the presence of HAA in blood is determined by an autosomal recessive trait [2,25,56]. In view of the following considerations, some of these interpretations may be rejected in favor of those that stress environmental factors in transmission and persistence of disease.

Down's syndrome: Blumberg and co-workers in 1967 [4] found that approximately 30 per cent of eighty-four patients with Down's syndrome had HAA in their serum. This was the highest frequency they had found in any of the different categories of patients tested. Subsequent studies by Sutnick et al. [55] indicated that the frequency of HAA in Down's syndrome was highest in patients in large institutions (20 to 30 per cent), was relatively low in patients in small institutions (3 per cent) and was not detected in noninstitutionalized patients with the same disorder. Con-

trols, who were similarly retarded patients in the same institutions, had a 2 to 5 per cent frequency of HAA. The patient's with Down's syndrome who had HAA in their serum generally had mildly elevated SGPT levels but normal bilirubin levels and other liver function tests; those who underwent biopsy showed histologic evidence of chronic viral hepatitis [55]. These findings were interpreted as indicating that patients with Down's syndrome were uniquely susceptible to hepatitis and that the hepatitis was transmitted orally, more in larger institutions, in which the environment seemed less hygienic, than in smaller institutions. They concluded that an environmental as well as a genetic, perhaps immunologic, host factor was involved.

Krugman and Giles [34] did not find a higher frequency of HAA in adult patients with Down's syndrome than in other patients of the same age group with similar mental retardation. They considered that the higher frequency of HAA in children with Down's syndrome resulted from their being admitted at an earlier age than other mentally retarded children and being more ambulatory. Hence they were exposed to environmental factors responsible for transmitting hepatitis over a longer period than most patients. They also suggested that studies by Blumberg et al. which were based on a single bleeding might not reflect accurately the over-all epidemiology of hepatitis in the institutions that were studied. Epidemiologic studies carried out over a long period by Szmuness and co-workers [57] on institutional transmission of HAA-positive viral hepatitis in patients with Down's syndrome and other forms of mental retardation led them to conclude, as did Krugman and Giles [34], that the occurrence, distribution and persistence of HAA in institutionalized mentally retarded patients of all types is influenced more by the age at which patients are exposed and the duration of exposure than by the etiology of mental retardation [57].

Lepromatous leprosy: Blumberg suggests that patients with lepromatous leprosy have a high frequency of HAA because of an abnormal immune response, possibly genetically determined, that predisposes them to a number of illnesses including viral hepatitis, lepromatous leprosy and leukemia [3,25,54]. The frequency of HAA in 584 patients with lepromatous leprosy was 9.4 per cent and in seventy-one patients with "borderline" lepromatous leprosy 9.9 per cent; whereas in 377 patients with tuberculoid leprosy it was 3.4 per cent and in 764 apparently normal persons in the nearby population 4.8 per cent [3]. These differences were considered to be significant despite the high frequency of HAA in the control population. Moreover, almost all the lepromatous leprosy patients were confined to a leprosarium, whereas the patients with tuberculoid leprosy were almost all outpatients.

It is well known that HAA is prevalent in institutionalized patients with Down's syndrome but not in outpatients. Just as in the case of institutionalized mentally retarded patients, the prevalence of HAA in institutionalized lepromatous leprosy cases was the highest in the youngest age group, suggesting, as in Down's syndrome, that age of exposure, intimacy of exposure and duration of exposure to endemic hepatitis are more important than genetic or immunologic factors in determining the prevalence of HAA.

Renal dialysis: Patients receiving long-term hemodialysis have been cited as examples of people with impaired immune mechanisms that alter the usual manifestations of hepatitis [54]. Of seventeen patients on maintenance hemodialysis studied by Turner and White [38], eight eventually had definite positive tests for HAA. Two of the eight had no clinical evidence of hepatitis; four had anicteric hepatitis; and two had the usual manifestations of symptomatic hepatitis with icterus. None were receiving immunosuppressive therapy. Over the two and a half year period that these cases were collected, hepatitis developed in eleven nurses and technicians on the staff of the hemodialysis unit, and seven of the nine whose blood was available within the first two weeks after onset had HAA in their serum. In ten of the eleven affected staff members typical hepatitis developed with elevated bilirubin levels in contrast to the chiefly asymptomatic and anicteric hepatitis of the patients on dialysis. The positive tests for HAA in the dialysis patients persisted as long as tested from four months to over two years, whereas HAA disappeared within four weeks from the blood of staff members with hepatitis. Blumberg and co-workers [54] also observed anicteric hepatitis with persistent HAA in eight patients receiving long-term hemodialysis, but acute viral hepatitis with icterus and transient HAA developed in six staff members of the dialysis unit. Thus, in two studies, in patients with uremia on long-term dialysis anicteric hepatitis developed with persistence of HAA whereas normal subjects who contracted hepatitis through intimate contact with the uremic patients had typical acute hepatitis with transient HAA. The source of hepatitis in some of the patients was no doubt transfused blood; however, others may have received low doses of virus parenterally or orally through contamination. Whether staff members were infected parenterally or otherwise is not known. Since severity of disease and duration of HAA may be related to dose of infectious agent (see section on transmission), the different manifestations of hepatitis in patients and staff may be attributable just as much to differences in dose of infectious agent or possibly to route of infection as to host response. It should be mentioned that most dialysis units do not have a problem with hepatitis in patients or staff.

Inherited traits: The early studies on inheritance of HAA, carried out when the antigen was considered to be a protein allotype, called Au(1), showed segregation of the antigen in families that was consistent

with simple autosomal recessive inheritance [2]. Later statistical analysis of the occurrence of HAA in family members of a different population showed highly significant agreement with the same genetic hypothesis [56]. Since much information suggested that HAA was linked to an infectious agent, the findings were considered compatible with an inherited susceptibility to a number of chronic infections including the agent that prouced HAA. A predominance of the antigen in male subjects and decreased prevalence in the older age groups were explained by factors which might affect susceptibility and resistance. The gel diffusion technic that was used to detect HAA in the genetic studies is relatively insensitive. It is apparent that observed values for HAA-positive family members would be two to three times higher if the more sensitive complement fixation [14, 58], or counterelectrophoretic technics [51,52], were used and that these values would not agree so closely with calculated theoretic values for autosomal recessive inheritance. Studies should be performed with more sensitive tests for HAA to see whether the evidence for recessive inheritance of susceptibility to hepatitis is upheld.

Clinical Value of Tests for HAA. For differential diagnosis: Since HAA is a specific manifestation of the agent of long-incubation hepatitis, the test for HAA can be used to differentiate the two major types of viral hepatitis. A positive HAA test defines the form of hepatitis precisely as "HAA-positive hepatitis." However, a diagnosis of "HAA-negative hepatitis" cannot be made on the basis of a negative HAA test, for the antigen may be present transiently or at a level too low to be detected by current methods of measurement. A patient can be assumed to have HAA-negative hepatitis only if it is known that the incubation period is short. The terms "serum" or "infectious" seem to be ambiguous in describing hepatitis and probably should not be used.

In any obscure or chronic liver disease the test for HAA may, if positive, be valuable in establishing a diagnosis for variants of the usual clinical manifestations of long-incubation hepatitis can be deceptive. Moreover, at least one diagnostic category of chronic liver disease, CAH, which is based chiefly on hepatic histology, can now be subdivided into "HAA-positive cases" that are most likely sequelae of long-incubation hepatitis and "HAA-negative cases" which may or may not be a consequence of viral hepatitis.

For prognosis: In keeping with the general impression that long-incubation hepatitis is usually more severe than short-incubation hepatitis, Cossart and Vahrmam [28] found that 90 per cent of their patients with HAA-positive hepatitis had a more prolonged hospital course with SGOT remaining usually above 100 units until the eighth week, compared to those with HAA-negative hepatitis who usually had SGOT values below 100 units by the fifth week. Sequential tests during acute hepatitis may be of further help in prognosis, for unusually long persistence of HAA indicates progression to chronic hepatitis whether or not symptoms subside and other laboratory tests become normal. Differentiation of CAH cases into those that are "HAA-positive" and those that are "HAA-negative" but have positive lupus erythematosus tests may prove to be valuable prognostically and therapeutically.

For prophylaxis: Since pooled normal gamma globulin is effective prophylactically in short-incubation (HAA-negative) hepatitis, but offers little or no protection against long-incubation (HAA-positive) hepatitis [33,34,59], documentation of HAA in a case of acute hepatitis would obviate the need for giving prophylactic gamma globulin to the patient's contacts. Also, identifying the type of hepatitis establishes the specificity of immunity that will develop; there is a high degree of immunity, although not absolute, to the homologous virus but no cross immunity between long-incubation (HAA-positive) and short-incubation (HAA-negative) hepatitis [34–36]. Highly sensitive tests specific for anti-HAA that are currently under investigation (see section on immunity) may permit identifying specificity of prior hepatitis infections.

Tests for HAA may be used to screen patients and medical staff in situations that predispose to endemic or frequent iatrogenic hepatitis, e.g., renal dialysis units, institutions for mentally retarded, groups of patients given multiple transfusions; for knowledge of the carrier state may help prevent contact cases. For the same reason, those in a position to transmit hepatitis, such as food handlers, surgeons and dentists should probably be tested for HAA. The most important application of screening tests is identification of blood donors who are carriers of HAA; all evidence indicates that HAA-positive blood transmits hepatitis (see section on transmission).

Currently available tests for HAA and anti-HAA are valuable, within the limits of their sensitivity, for all of these purposes, realizing that only positive tests are significant. The tests are simple and inexpensive, and should be employed as routine laboratory procedures in hospitals and blood banks.

RELATIONSHIP BETWEEN HAA AND TRANSMISSION OF HEPATITIS

There is no direct evidence that HAA is an infectious agent. Physical and chemical characteristics of the antigenic material suggest that it is chiefly noninfectious empty virus coat, but the amounts of HAA that circulate are so great that only a small fraction would have to be infectious virion to approach the blood concentration of virus characteristic of other viral illnesses. As yet there is no known laboratory animal that can be used for passing the agent, and appropri-

TABLE V HAA in Subhuman Primates

Animal	No. Tested	No. HAA+
Chimpanzees	215	7
Orangutans	52	3
Gibbons	23	3
Baboons	56	0
Rhesus monkeys	176	0
Vervets	139	0
Marmosets	30	0

ate conditions for propagating the virus in tissue culture have not been found. Therefore, all information on transmission of viral hepatitis has been derived from clinical observations and human experimentation.

HAA in Animals. Outbreaks of hepatitis among handlers of higher apes and occurrence of a disease similar to human hepatitis in chimpanzees have implicated apes as hepatitis carriers [60,61]. Apparently normal chimpanzees and other higher apes have a relatively high frequency of HAA in their blood (Table V) [8,29,62,63], and seven chimpanzees that were carriers of HAA all had histologic evidence of mild active hepatitis on biopsy. Antigen that is immunologically indistinguishable from HAA found in human serum was detected in seven chimpanzees, three orangutans and three gibbons but not in several species of monkeys [8,62], although others have mentioned the presence of HAA in monkeys [25]. Marmoset monkeys have been used as a possible animal model for human viral hepatitis [64], but even those animals with liver lesions after inoculations with blood from hepatitis patients have not been found to have HAA in their serum [29]. According to a recent report [65] marmosets may be susceptible to infection with short-incubation hepatitis virus rather than the type that produces HAA. It is not known whether HAA-positive hepatitis is a natural infection of higher apes or whether they acquire the infection from contact with man or through the common practice of animal dealers giving infant apes transfusions of human blood.

Apparent Transmission of Hepatitis by HAA-positive Transfusions. Okochi and Murakami [5] noted the development of HAA-positive hepatitis in three recipients of antigen-positive blood, but also found the antigen in six post-transfusion hepatitis patients who had not received antigen-positive blood. Failure to detect HAA in the transfused blood was considered to be a reflection of the insensitivity of the agar gel technic. Gocke et al. [31], in a retrospective study of twelve patients who had received antigen-positive blood, found that hepatitis developed in nine and that serum from seven of the nine was positive for HAA. More recently Gocke [16] has found that the frequency of hepatitis in forty patients given transfusions of blood containing HAA was approximately 70 per cent [66], whereas only four cases of hepatitis were seen in sixty-nine recipients of antigen-negative blood [31].

Experimental Transmission of Hepatitis by HAA-positive Material. Giles et al. [30] and Krugman and Giles [34] demonstrated the regular appearance of HAA-positive hepatitis in recipients of HAA-positive serum obtained from a patient during the acute phase of long-incubation hepatitis. They also observed apparent "second passage" of HAA in two subjects who received serum from a donor who had been inoculated with the MS-2 agent and had been an asymp-

TABLE VI Frequency of Hepatitis and HAA in Recipients of Blood Products Containing HAA

Inoculated Material	HAA Titer by CF	Subjects Inoculated No.	Hepatitis Cases		Not 111		Total HAA+
			No.	HAA+	No.	HAA+	
Acute phase serum*	1:1,280	12	3	3	9	5	8
Serum from a donor whose blood caused hepatitis†	1:1,280	14	9	8	5	1	9
Serum from same donor 10 mo later	Unknown	4	2	2	2	0	2
Human thrombin‡	1:10	6	3	2	3	2	4

NOTE: Inoculation was 1 ml subcutaneously. Each of the inoculated materials, when concentrated on a cesium chloride gradient [9], were found to contain virus-like particles characteristic of HAA on electronmicroscopy. Data are taken from Barker et al. [41].

* Acute phase serum was obtained ten days after appearance of clinical symptoms and icterus in a subject inoculated ninety-four days prior to that time with an icterogenic plasma pool that contained HAA by immunologic and electronmicrographic criteria.

† The donor was suspected of being a hepatitis carrier because hepatitis developed in a recipient of his blood, although the donor had no history of liver disease.

‡ The thrombin was suspected of transmitting hepatitis after clinical use.

TABLE VII Frequency of Hepatitis and HAA in Recipients of Logarithmic Dilutions of Plasma Containing HAA

Plasma Dilution	Subjects Inoculated	Hepatitis Cases		Not Sick		Total HAA+
		No.	HAA+	No.	HAA+	
10^{0}	37	22	20	15	5	25
10^{-3}	5	2	2	3	1	3
10^{-4}	5	1	1	4	2	3
10^{-5}	5	0	0	5	2	2
10^{-6}	5	0	0	5	3	3
10^{-7}	5	0	0	5	2	2
10^{-8}	5	0	0	5	0	0

NOTE: Inoculations were 1 ml subcutaneously. The titer of HAA in undiluted plasma was 1:10 by complement fixation; HAA could not be detected in the undiluted plasma by agar gel diffusion, but virus-like particles typical of HAA were seen on electronmicroscopy after the plasma was concentrated on a cesium chloride gradient. Data are taken from Barker et al. [41].

tomatic carrier of HAA for almost four years after inoculation.

In studies involving over 100 recipients, Barker et al. [41] showed that plasma and a purified plasma protein that contained HAA transmitted HAA-positive hepatitis (Table VI). The materials that produced hepatitis and the weekly serum samples obtained from inoculated subjects had been stored at —20°C from the time of the transmission studies in 1951 to 1954 [22] until analyzed for HAA in 1969 [41]. HAA and virus-like particles characteristic of HAA were present in the inoculated materials. Most of the recipients in whom hepatitis developed also had HAA in their blood during the incubation period and acute phase of the disease, and many recipients in whom clinical or laboratory evidence of hepatitis did not develop nevertheless had HAA in their serum. HAA appeared in the asymptomatic recipients only after a delay consistent with the incubation period of hepatitis. The experiment with acute phase serum (Table VI) is a documented second passage of HAA-positive hepatitis with serum from an HAA-positive donor in whom hepatitis had developed after inoculation with an HAA-positive plasma pool.

Relationship between Viral Dose, Mode of Transmission and Severity of Hepatitis. Studies shown in Table VII [41] and additional observations by Barker et al. [42] indicate that recipients of low doses of infectious virus (plasma dilutions 10^{-5} to 10^{-7} in Table VII) have a mild illness that is anicteric and frequently subclinical, have hepatitis and HAA after an unusually long incubation period and tend to retain the antigen indefinitely. Of twelve subjects who became persistent carriers of HAA after participation in hepatitis transmission studies, eight had minimal symptoms of hepatitis and bilirubin elevations no greater than 2 mg, and the remaining four had no symptoms and no laboratory abnormalities [42]. Others [34,67] have also observed prolongation of the incubation period of hepatitis after the administration of low infectious doses and noted that the hepatitis that developed was usually mild and anicteric. These observations imply that, conversely, those in whom mild or subclinical hepatitis develops and who retain HAA for unusually long periods most likely were infected by relatively low doses of virus.

In subjects prone to the development of persistent circulating HAA without significant liver disease, viz., patients with Down's syndrome [34,38,39,55,57], or lepromatous leprosy [3,25,54], hemodialysis patients [38,39] and carriers in tropical populations in whom HAA is prevalent [2,56], may all have been infected by low doses of virus and may not necessarily have unusual susceptibility to hepatitis. Additional observations relating to mode of transmission, severity of disease and apparent infectious dose are those of Mirick and Shank [37] that epidemic long-incubation hepatitis which appeared to spread by person to person contact was usually anicteric, and those of Krugman [34] and Szmuness [57] et al. that institutional transmission of HAA-positive hepatitis by apparent contact requires a long period of exposure, suggesting low infectivity.

It is apparent from the dilution studies in Table VII that if HAA is the virus particle responsible for hepatitis, the minimum amount which can transmit hepatitis is far below that which is detectable by the most sensitive tests for HAA currently available. HAA in the undiluted icterogenic plasma pool had a titer of 1:10 by complement fixation; virus-like particles were visible by electronmicroscopy only after the plasma was concentrated on a cesium chloride gradient; and the undiluted plasma gave a negative test for HAA in agar gel. Nevertheless, overt clinical HAA-positive hepatitis was transmitted by 1 ml of a 1:10,000 dilution of the plasma, and apparent subclinical hepatitis as judged by replication of HAA was transmitted by 1 ml of plasma diluted as high as 1:10,000,000. Compared to the most sensitive laboratory tests currently available, the biologic test for HAA by transmission of hepatitis in man is more sensitive by a factor of approximately 10^{6}.

The high infectivity of virus by the parenteral route suggests that hepatitis can be caused by amounts of blood that could be transferred by hematophagous insects. Insect vectors, whether bedbug, louse or mosquito, could account for transmission that appears to involve person to person contact in institutions or in tropical regions in which HAA carriers are prevalent. Adding weight to this possibility are the recent findings by Prince [68] that in some tropical populations from Africa, Asia and South America where HAA is ten to a hundred times more prevalent than in

New York, there is no significant difference in the prevalence of HAA between male and female subjects and no apparent decline with increasing age in the frequency of serum containing antigen.

Relationships Between Perinatal and Maternal HAA-Positive Hepatitis. There is no definite information on transplacental transmission of HAA-positive hepatitis. Three patients with perinatal hepatitis whose mothers had HAA-positive hepatitis during pregnancy were found to have HAA in their blood [69]. The cord blood of two infants whose mothers were HAA-positive at the time of delivery was negative for antigen; cord blood in the other infant was not tested. Transplacental transmission seems less likely than infection of the infant shortly after birth.

Attempts to Identify HAA in Body Fluids Other than Blood. In view of the apparent transmission of long-incubation hepatitis by person to person contact under some circumstances, the possibility has been considered that infection may occur via the fecal-oral route, through urine or through pharyngeal secretions. However, early attempts to identify the agent of long-incubation hepatitis in feces and in nasopharyngeal secretions by human transmission studies were not successful [35,36]. Recent attempts to measure HAA directly in various body fluids also have not been successful. Patients with HAA in their blood that could be measured at dilutions greater than 1:2,000 by complement fixation technics did not have detectable HAA in saliva concentrated tenfold, in urine concentrated a hundredfold or in 20 per cent stool extracts [70].

Although HAA was not found in extracts of stool from patients who had HAA in their serum, a different protein antigen associated with hepatitis was found in these extracts [71]. The new antigen was precipitated in agar gel diffusion and electrophoretic tests by serum from 10 to 15 per cent of patients receiving multiple transfusions and from some patients with HAA-positive acute viral hepatitis. The antigen was found in extracts of stool from five of five patients with acute viral hepatitis, two of five patients with chronic hepatitis and one patient who had received multiple transfusions, but not in stool extracts from twenty-five normal subjects and patients without liver disease. As shown in Figure 6, the material in stools does not appear to share any antigenic determinants with HAA, although half of the serums that precipitated the stool antigen contained anti-HAA. The antigen appeared to be a protein of molecular weight >300,000. Because of the association with hepatitis the new antigen could be an altered component of the virus that produces HAA, or a different viral agent that may complement or accompany that virus. A detailed report on the new antigen is forthcoming [71].

HAA in Plasma Fractions. All of the Cohn cold ethanol plasma fractions except for fraction II contain measurable amounts of HAA if the unfractionated plasma is heavily contaminated with antigen (Table I) [24]. These findings corroborate the extensive clinical experience indicating that fraction II (pooled normal gamma globulin) does not transmit hepatitis, whereas other fractions such as I (fibrinogen) and III (thrombin) are implicated often. Fraction II probably does not contain significant amounts of specific antibody against HAA; attempts to neutralize the long-incubation hepatitis agent with fraction II [34] or to measure anti-HAA in fraction II [24] have not been successful. Fraction V (albumin) contains relatively small amounts of HAA, and HAA in albumin remains immunologically stable by precipitation tests after the albumin has been heated at 60°C for ten hours [24]. Since heated albumin does not transmit serum hepatitis, simply finding a positive test for HAA does not necessarily indicate infectivity. On the other hand, failure to find HAA in a plasma fraction does not give assurance that the fraction is free of infectious agent; many commercial fraction I preparations known to have transmitted long-incubation hepatitis [72] do not contain detectable amounts of antigen by agar gel or complement fixation technics [8,20]. It can be assumed that most pools of plasma prepared commercially, and fractions from pooled plasma except for gamma globulin, contain sufficient virus to transmit hepatitis but insufficient HAA to be detected by in vitro tests, as in transmission experiments shown in Table VII.

Detection of HAA Carriers Among Blood Donors. Prevalence of HAA in different blood donor populations measured by the insensitive agar gel diffusion

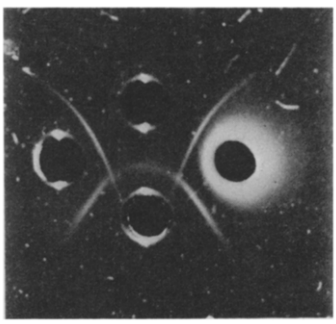

Figure 6. Agarose diffusion pattern. Stool extract from patient with HAA-positive acute hepatitis in lower well; stool-precipitating antibody and anti-HAA in upper well; and in left and right wells, serum from two different patients with acute viral hepatitis containing high titer HAA and stool-precipitating antibody but no measurable anti-HAA. The HAA in the left and right wells reacted with anti-HAA in the upper well; the stool extract in the lower well reacted with the stool-precipitating antibody in the other three wells. Lines of the HAA precipitate and stool precipitate cross without evidence of partial identity.

technic is shown in Table III. Complement fixation technics are at least a hundredfold more sensitive than agar gel diffusion [20] and detect approximately twice as many HAA carriers among blood donors [58]. It has been estimated that the insensitive agar gel diffusion technic detects 20 to 40 per cent of the carrier population among blood donors [51]. If these estimates are correct, then by screening with complement fixation it should be possible to eliminate at least 40 to 80 per cent of donors who transmit hepatitis. In our experience the counterelectrophoretic technic [51,52] which is approximately ten times more sensitive than agar gel diffusion, but ten- to twenty-fold less sensitive than complement fixation, detects the same HAA carriers as complement fixation technics. Thus it appears that most HAA carriers are detectable by a technic only ten times more sensitive than simple agar gel diffusion, and the additional sensitivity of complement fixation technics does not appear to reveal a significantly greater number. Although further study is necessary, indications are that carriers tend to have levels of HAA that are easily detected by currently available simple and inexpensive tests. Screening donors for HAA by technics equivalent in sensitivity to counterelectrophoresis or complement fixation seems to be a necessary function of blood banks, for the association between the presence of HAA and transmission of hepatitis is conclusive.

ANTI-HAA, ANTIGEN-ANTIBODY COMPLEXES AND IMMUNITY

Anamnestic Antibodies. The antibodies used diagnostically to measure HAA by precipitin and complement fixation technics all come from patients who have received multiple transfusions and in whom high titer "hyperimmune" anti-HAA developed apparently as a result of repeated exposure to antigen in transfused blood. Okochi and Murakami [5] using an agar gel precipitin technic observed fifteen instances in which anti-HAA appeared after transfusions of HAA-positive blood and an additional seven instances in which anti-HAA appeared following transfusion of blood in which HAA was not found, a circumstance considered to reflect the insensitivity of the method of measuring HAA. In fourteen of the patients antibody developed within two weeks and in six patients between two and four weeks after transfusion; in the remaining two patients antibody was detected fifty-six and sixty days after transfusion, respectively. Development of relatively high titer anti-HAA shortly after transfusion suggests that antibody response was anamnestic; for, as shown in Figure 4, detectable HAA does not usually appear in blood until five weeks or more after inoculation of infectious plasma. Whether or not the hepatitis developed in the patients who received these transfusions or whether or not they had a history of prior hepatitis or transfusion was not mentioned in the study by Okochi and Murakami. Others have noted apparent anamnestic appearance of complement-fixing anti-HAA within two weeks after transfusion [29,74]; and Krugman and Giles [34] also observed occasionally low titer complement fixation anti-HAA in patients who had been re-exposed to HAA-positive serum a year after an initial exposure. These various observations suggest that antibodies measurable by agar gel precipitin or complement fixation technics arise commonly under clinical conditions that permit an anamnestic response, but antibodies of this type can rarely be detected during the course of a primary, acute or chronic hepatitis infection.

Anti-HAA in Acute and Chronic Hepatitis. In none of the many cases of acute and chronic hepatitis studied by Wright et al. [27] (Tables II and IV), did anti-HAA detectable by agar gel precipitin technics develop. Cossart and Vahrman [28] did not find antibodies by complement fixation technics in serum obtained during the convalescent state from two patients with HAA-positive acute hepatitis using HAA that had been present in the homologous serum as antigen. Krugman and Giles [34] did not find anti-HAA by complement fixation after primary acute HAA-positive hepatitis in a large series of experimentally infected patients. Shulman et al. [29] tested serum obtained at weekly intervals from twenty-two patients with HAA-positive hepatitis during a one to two month period after onset of disease and found only one antibody detectable by agar gel precipitin technics and two antibodies of low titer detectable only by complement fixation. None of sixty additional patients from whom one or two random samples were obtained during the recovery phase of acute hepatitis or during chronic hepatitis had antibody by complement fixation or agar gel precipitin technics. However, with a recently developed highly sensitive hemagglutination test for anti-HAA, many of the same eighty-two patients with acute and chronic hepatitis were found to have antibodies (Table VIII).

The hemagglutination test, which involves coating red cells with isolated HAA and agglutinating these cells with anti-HAA, is approximately 2,000-fold more sensitive than agar gel precipitin tests and approximately a hundredfold more sensitive than complement fixation tests for measuring antibody. With the hemagglutination test the frequency of anti-HAA formation in patients recovering from HAA-positive acute hepatitis was approximately 20 per cent; in patients with prolonged hepatitis, 43 per cent; and in chronic active hepatitis, about 17 per cent. That antibody was detected in three of four cases of hepatitis associated with drug use may reflect anamnestic response to intermittent reexposure to antigen. The presence of anti-HAA did not seem to influence the course or severity of disease.

Significance of Antigen-Antibody Complexes. Of

special note is the finding that HAA occasionally can be measured in the presence of anti-HAA. In the patient recovering from acute hepatitis (Table VIII), HAA was measured at a relatively low titer (1:10 by complement fixation) in face of a relatively high titer antibody (1:40 by complement fixation). This circumstance may reflect readily reversible antigen-antibody complexes involving a high concentration of low avidity antibody. The other two instances of HAA being measured in the presence of anti-HAA were in cases of chronic active hepatitis. Both of these antibodies were low titer (1:10 and 1:20, respectively, by the hemagglutination technic) and HAA measured in the presence of these antibodies was in one instance low titer (1:20 by complement fixation) and in the other instance high titer (1:2000 by complement fixation). Antigen coated red cells used in the hemagglutination test are effective in competing with antigen-antibody complexes for anti-HAA probably because the cell agglutinate that forms favors the competitive reaction.

Although antigen and antibody could not be measured directly in many patients, the presence of antigen-antibody complexes was often reflected by anticomplementary activity [20,29]. Anticomplementary activity is the ability of serum alone without further addition of antigen or antibody to inactivate complement. An example of the characteristic temporal relationship between development of anticomplementary activity and appearance of circulating HAA that occurred in three patients in whom hepatitis developed after receiving a transfusion is shown in Figure 7. These findings suggested that antigen-antibody complexes capable of fixing complement can appear early in the course of the disease, can no longer fix complement when antigen is in excess, and can regain the ability to fix complement as the antigen to antibody ratio decreases. Evidence that the anticomplementary activity is caused by antigen-antibody complexes is the presence of virus-like particles in anticomplementary serum, the neutralization of anticomplementary activity by addition of excess purified HAA which appears to change complexes into non-complement fixing antigen-excess forms and the ability of organic solvents which reverse antigen-antibody complexes to decrease anticomplementary activity and permit immunologic detection of HAA in the treated serum [20].

Almeida and Waterson [76] have interpreted electronmicrographs of serum specimens obtained from patients with different forms of HAA-positive hepatitis as showing either randomly distributed particles indicative of free antigen or clumped particles suggesting immune complexes. They considered that it was possible to differentiate by electronmicroscopy between complexes formed in antigen or in antibody excess. Recently Millman and co-workers [77] also obtained evidence by an immunodiffusion technic

TABLE VIII Anti-HAA in Acute and Chronic Hepatitis Measured by Hemagglutination

Diagnosis	Cases (no.)	Anti-HAA Total (no.)	Anti-HAA HAA Present (no.)	No Antibody Total (no.)	No Antibody HAA Present (no.)
Long-incubation hepatitis recovery phase*	22	4	1	18	5
Undifferentiated viral hepatitis recovery phase	8	2	0	6	1
"Infectious" hepatitis recovery phase	6	0	0	6	0
Prolonged viral hepatitis	14	6	0	8	0
Chronic active hepatitis	24	4	2	20	8
Hepatitis in drug users	4	3	0	1	0
Cholestatic hepatitis	1	0	0	1	0
Cirrhosis	3	1	0	2	0
Total	82	20	3	62	14

NOTE: Only one antibody was detected by agar gel diffusion; this antibody had a complement fixation titer of 1:40; two additional antibodies were detected at a titer of 1:5 by complement fixation but not by agar gel diffusion. The seventeen other antibodies were detected only by hemagglutination assay. The titers by hemagglutination varied from 1:16 to 1:128. See section on "Measurement of HAA and Anti-HAA" for comparison of sensitivity of techniques.

* Weekly samples were obtained on each patient for one to two months after onset of symptoms. In all other categories one or two random serum samples were obtained.

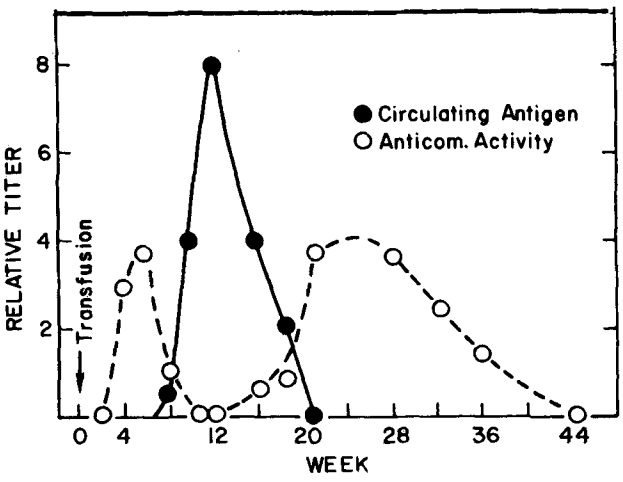

Figure 7. Serum samples are from a patient in whom hepatitis developed after receiving a transfusion. Antigen was measured by quantitative complement fixation with anti-HAA. Anticomplementary activity was the amount of complement fixed by the patient's serum without additional reagents. From Shulman et al. (Ann Intern Med 72: 257, 1970) [29].

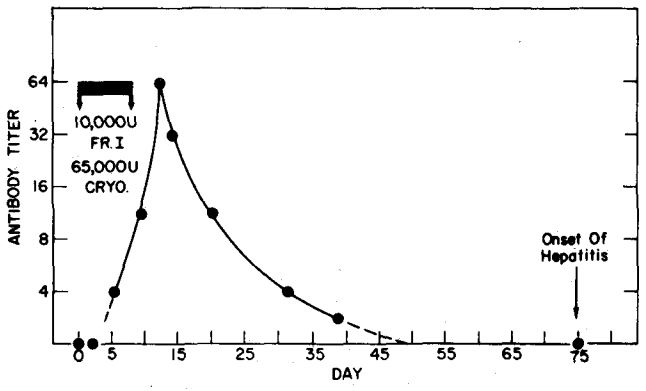

Figure 8. *Antibody titer is the reciprocal of the maximum dilution of serum that fixed more than 2 units of complement in the presence of optimum antigen. Treatment was required for hemorrhage caused by an idiopathic anti-factor VIII.*

that HAA and anti-HAA can be present together in the serum of patients with viral hepatitis.

The presence of antigen-antibody complexes in some patients with viral hepatitis and depression of complement levels in some instances during the acute phase of illness suggest that certain of the multisystem manifestations of hepatitis may be immune in nature. For example, acute arthralgia or arthritis, myalgia, rash and urticaria, generalized vasculities and hyperglobulinemia which may accompany acute or chronic viral hepatitis, may all be manifestations of circulating antigen-antibody complexes. Bulkley et al. [48] found a high frequency of arthritis and arthralgias among patients with chronic active hepatitis whose serum was positive for HAA. Alpert et al. [78] found that serum complement levels in patients with acute hepatitis were depressed in those who had arthralgias or arthritis associated with either fever or urticaria and circulating HAA. Gocke et al. [79] observed four patients with polyarthritis who had circulating HAA and, on the basis of immunofluorescent studies, possibly deposits of HAA complexed with antibody in small blood vessels.

Immunity and Possibilities of Immunotherapy. It is known that attacks of acute viral hepatitis confer a high degree of immunity to the homologous form of hepatitis [34–36]; but immunity is not absolute; and as indicated by the following observations, it cannot be correlated with anti-HAA levels. During the incubation period of hepatitis antibody can arise without preventing further development of HAA or progression of disease (Figure 7). Hepatitis can even follow marked anamnestic antibody responses (Figure 8), but under these circumstances the disease is mild. The patient whose data appear in Figure 8 was treated with cryoprecipitate from 250 donors over a one week period. Within five days after the start of therapy, anti-HAA appeared in the patient's serum and reached peak titer within two weeks. Approximately two months later, when antibody had decayed, anicteric mild hepatitis developed with elevation of SGOT and SGPT levels [29]. Krugman reported a similar case [34] of accidental reinfection with HAA-positive material that elicited transient anti-HAA response followed by anicteric mild hepatitis with elevation of SGOT values and circulating HAA. Two similar instances of hepatitis following what appeared to be anamnestic antibody responses have also been reported by Holland et al. [74]. These two patients also had mild anicteric hepatitis after decay of anti-HAA that had appeared within two weeks after transfusion. They described a third patient with more severe hepatitis in the presence of high titer anti-HAA, but cytomegalovirus infection could not be ruled out. These various observations suggest that the usual anti-HAA is probably not an effective neutralizing antibody. It is not clear whether immunity is due in part to antibody or entirely to the thymus-dependent system, for example as in measles [80]. Pooled normal gamma globulin does not seem to protect against HAA-positive hepatitis compared to its high degree of effectiveness in protecting against short-incubation hepatitis, but it may modify the severity of the disease [59].

The finding of circulating complexes of antibody with viral antigen is novel in human disease, but circulating virus-antibody complexes are well documented in animals. For example, in mice, lactic dehydrogenase virus (LDV) stimulates antibodies that combine with virus, but virus-antibody complexes remain infectious and the animals have lifelong viremia in the presence of anti-LDV [81]. The LDV-antibody complex is relatively resistant to neutralization with antibodies, and the complex transmits the disease when inoculated into other animals [81]. Moreover, heterologous, non-neutralizing antibodies are elicited by certain viruses; for example, rabbit anti-herpes simplex virus (HSV) which forms a complex with the virus but does not prevent viral infectivity [82]. As in the case of complexes of LDV and HSV with antibody, complexes of HAA with anti-HAA appear to retain infectivity. Persistence of HAA and low grade hepatitis following low doses of virus, and development of chronic hepatitis following acute infections may involve variations in the immune response, but at present this cannot be assessed.

The possibility of using anti-HAA therapeutically in severe HAA-positive hepatitis is attractive; but, in view of the following considerations, it would be difficult to predict the outcome. Levels of HAA cannot be correlated with severity of illness, most human anti-HAA antiserum does not appear to neutralize infectivity, intracellular virus would not be affected by antibody, large amounts of antibody would be needed to bind the usual amounts of HAA that circulate, and potentially complexes of HAA with anti-HAA would be harmful. Experimental use of antise-

rum appears to be justified only as a last resort. Use of anti-HAA experimentally as a prophylactic agent in high risk patients seems reasonable. Although pooled normal gamma globulin does not prevent long-incubation hepatitis, the question of whether it ameliorates the disease is still open. Known doses of hyperimmune anti-HAA would permit critical evaluation of the possible protective effects of this antibody. Whole antiserum could not be used prophylactically because of the possibility of its containing infectious virus-antibody complexes, but purified gamma globulin should be free of virus. HAA used as a vaccine after physical or chemical treatment to inactivate infectious virus would no doubt elicit specific antibodies and perhaps confer some protection. However, partial protection, if equivalent to low dose infection, conceivably could predispose to the carrier state associated with low grade chronic hepatitis.

MEASUREMENT OF HAA AND ANTI-HAA

Methods. Agar gel diffusion: Ouchterlony gel diffusion was used by those who first defined HAA and is still the most commonly employed technic because it is the simplest. However, immunodiffusion has two disadvantages: it is relatively insensitive, and it is a slow test requiring one to three days for completion. Most antigenic serum will not produce precipitin lines if diluted more than 1:2 or 1:4 in immunodiffusion tests [20]. Only serum with unusually large amounts of antigen can be diluted as much as 1:16. Similarly, most serum containing antibodies cannot be used for detecting antigen if diluted more than 1:2 or 1:4. Figure 9 shows a typical immunodiffusion pattern with lines of identity between HAA samples from different sources.

The use of agarose rather than agar gel increases the sensitivity about twofold. Sensitivity can also be increased by concentrating the serum samples by dehydration or by repeated filling of the wells [29]. It has been estimated that immunodiffusion technics detect only 20 to 40 per cent of blood donors who are carriers of HAA [51].

Counterelectrophoresis in gel: Pesendorfer et al. [52] and Gocke and Howe [83] reported a simple electrophoretic technic which increases the sensitivity of the immunodiffusion test approximately tenfold and permits development of precipitin lines within hours rather than days. Prince and Burke [51] reported a similar test using somewhat higher voltage. The sensitivity of counterelectrophoresis is greater than immunodiffusion because the reactive components placed in opposite wells travel toward each other and concentrate at an interface rather than diffuse in all directions. Figure 10A shows titration by counterelectrophoresis of a serum containing HAA, and Figure 10B shows a simultaneous test for antigen or antibody suggested by Krassnitzky et al. [84] in which the patient's serum is placed between wells containing antigen and antibody. In Figure 10B, serum in positions 1, 2, 3 and 5 contains HAA and serum in position 6 contains anti-HAA, whereas that in position 4 contains neither.

The counterelectrophoresis test is rapid and practical. In our experience it detects twice as many carriers of HAA in blood donor groups as does immunodiffusion. Counterelectrophoresis is also helpful in detecting antibodies in some anticomplementary serums that contain antigen-antibody complexes and are difficult to assay in complement fixation systems.

Cellulose acetate immunoelectrophoresis: Saravis et al. [85], have reported electrophoresis of serum on cellulose acetate membranes impregnated with anti-HAA as a test for HAA. The presence of antigen is indicated by a precipitin pattern which forms with HAA-positive serum. This technic employs large amounts of antibody, is more difficult to perform than counterelectrophoresis, and is no more sensitive than immunodiffusion. The cellulose acetate test has no apparent advantages.

Complement fixation: Complement fixation procedures for measuring HAA appear to be the most sensitive and rapid available. This technic also has the unique advantage of being able to detect antigen-antibody complexes through measurements of anticomplementary activity. Complement fixation tests for measuring antigen or antibody quantitatively or qualitatively can be completed within two hours and are a hundred- to two hundredfold more sensitive than immunodiffusion technics for detecting antigen and twenty- to fortyfold more sensitive for detecting antibody. The complement fixation test for HAA and anti-HAA can be automated like other complement fixation tests.

Semiquantitative or qualitative complement fixation with microtiter plates, shown in Figure 11, can be

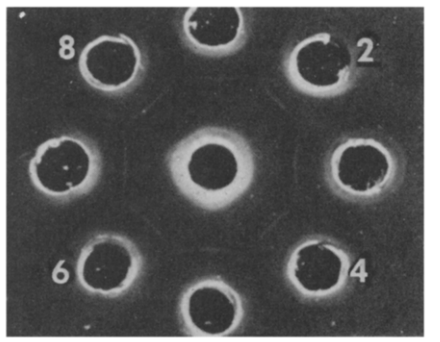

Figure 9. Lines of identity in agar gel. Anti-HAA in center well. Wells 1 and 3 contain serum from chimpanzees; wells 2 and 7 from patients diagnosed as having infectious hepatitis; well 5 from a gibbon; and wells 4, 6 and 8 from patients diagnosed as having serum hepatitis. The medium is 0.9 per cent agarose with 0.05 M veronal buffer pH 7.6.

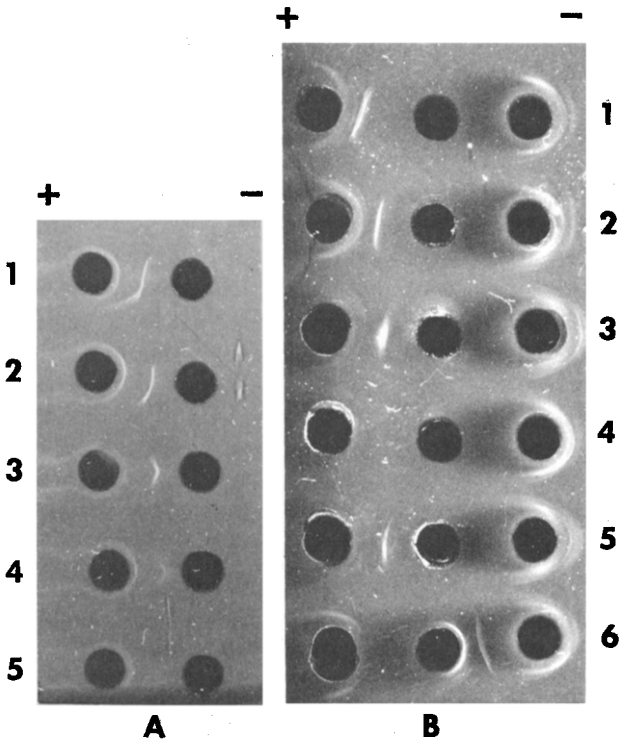

Figure 10. Titration of HAA by counterimmunoelectrophoresis. A, serial twofold dilutions of an HAA-positive serum are in the wells on the right near the cathode, beginning with a dilution of 1/2 in number 1. Anti-HAA is in the wells on the left near the anode. Wells contained 0.025 ml of serum. Electrophoresis was carried out at 300 V for 90 minutes. The last visible line is between the wells at the fourth level, a 1:16 dilution of serum. This serum containing antigen gave a precipitin line by agar gel diffusion only if used undiluted. B, patients' serum being tested is placed in the center wells, anti-HAA in wells near the anode on the left and a known sample of HAA in the wells on the right. Conditions are otherwise the same as in A. The lines between wells on the side near the anode at levels 1, 2, 3 and 5, are examples of patients' serum containing HAA. The line on the side of the cathode at level 6 is an example of an antibody against HAA in a patient's serum. Level 4 shows no reaction.

performed in a routine manner by laboratories using this method for other serologic tests. A recently developed qualitative complement fixation test in which measurement depends on diffusion of complement in agarose gel can be carried out as easily as immunodiffusion tests (Figure 12) [58]. The gel containing sensitized indicator cells can be stored for at least one week and supplied to laboratories not equipped to standarize reagents.

The various adaptations and modifications of complement fixation tests have approximately the same sensitivity. Although some workers use overnight incubation at 5°C to fix complement [86], this prolonged incubation is unnecessary for maximum complement fixation takes place in thirty to sixty minutes at 37°C.

Complement fixation technics are more sensitive than any other technic except hemagglutination for detecting HAA. Complement fixation tests and hemagglutination tests are equally sensitive for detecting HAA, but the complement fixation tests are simpler for this purpose. Although complement fixation tests are ten- to twentyfold more sensitive than counterelectrophoresis for detecting HAA, the titer of HAA in blood of most carriers is high enough to be detected by counterelectrophoresis. Availability of suitable antibodies might limit application of complement fixation for routine testing, for not all antiserums that precipitate HAA in agar gel are suitable for complement fixation [20]. However, high quality complement fixation antibodies can be produced in higher apes [20] and, in limited amounts, in guinea pigs [19].

Hemagglutination: A hemagglutination assay recently developed in this laboratory involves coating red cells with purified antigen using chromic chloride as a coupling agent [73]. Hemagglutination reactions were performed in microtiter plates and read by the pattern of agglutination shown in Figure 13. This test,

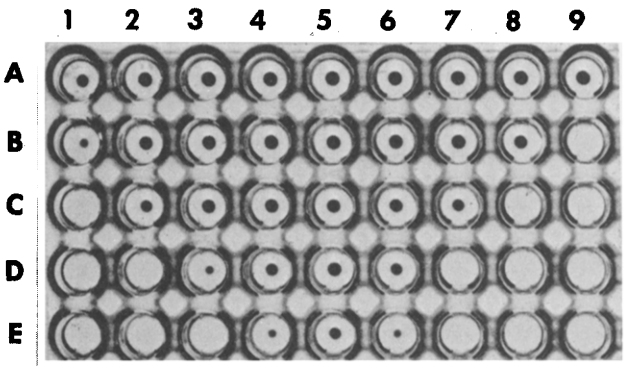

Figure 11. Complement fixation by box microtitration. Serial twofold dilutions of antibody and antigen were mixed with 2 units of complement in a total volume of 0.075 ml and incubated at 37°C for one hour before 0.025 ml of a 1 per cent suspension of sensitized sheep erythrocytes was added. There are nine different antigen dilutions, beginning at 1:8 in well 1, and ending at 1:2408 in well 9. Antiserum dilutions begin at 1:10 in well A, and end at 1:160 in well E. Complement fixation is indicated by the presence of unlysed red cells. Cells that are not lysed form buttons at the bottom of the wells. Antibody at 1:10 dilution in the A row fixes all the complement with each of the antigen dilutions; therefore none of the red cells are lysed. Antibody 1:20 is insufficient to fix all of the complement with the lowest dilution of antigen in well 1 (prozone effect), and the prozone becomes more marked with increasing dilutions of antibody. If weak antibody is used in complement fixation tests and a range of dilutions is not used, antigen excess can produce an apparent negative reaction.

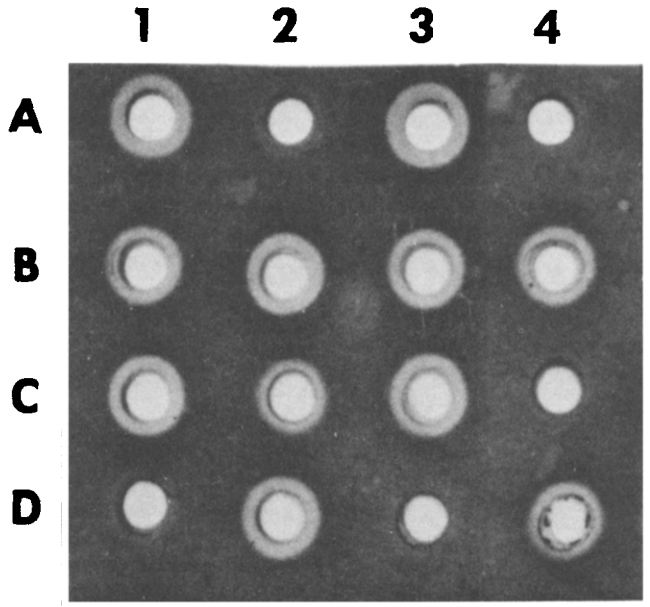

Figure 12. Patterns formed in a complement diffusion test. The wells are in agarose in which 0.5 per cent sensitized sheep erythrocytes are embedded. Reaction mixtures in microtiter plates consist of test serum incubated with known HAA or anti-HAA, and 1 to 2 units of complement in a total volume of 0.075 ml. After one hour at 37°C, 0.01 ml of mixture is transferred to the wells in agarose plates containing the indicator cells. After an additional one hour at 37°C, hemoglobin from complement-lysed cells diffuses to give a clear halo indicating lack of complement fixation, e.g., wells A1, A3 and all the B wells, etc. No halo indicates complement fixation, i.e., the presence of antigen if anti-HAA is used as a reagent or of antibody if HAA is used as a reagent. Positive complement fixation tests occurred in wells A2, A4, C4, D1 and D3. If anticomplementary activity is present, it appears in control wells as a positive complement fixation test.

including coating of cells with antigen, can be carried out in about two hours. Equipment and reagents, except for purified antigen, are readily available in blood banks. The purified antigen, prepared from HAA-positive plasma by a combination of rate and isopycnic banding in cesium chloride (CsCl) density gradients [13] is stable indefinitely at 5°C, and coated cells may be preserved for long-term use with glutaraldehyde [73].

Red cells with HAA attached are agglutinated by extremely small amounts of anti-HAA. The sensitivity of hemagglutination appears to be at least 2,000-fold greater than that of immunodiffusion, a hundredfold greater than that of complement fixation and approximately the same as that of a radioimmunoassay for measuring anti-HAA. In view of the simplicity of the hemagglutination assay compared to radioimmunoassay, the latter appears to be of little value. Data obtained by hemagglutination on anti-HAA formed during acute and chronic viral hepatitis is presented in Table VIII.

To detect antigen by hemagglutination, antibody that could produce agglutination at a dilution of 1:10 was mixed with an equal volume of serum suspected of containing HAA. Neutralization of antibody by antigenic serum prevented hemagglutination. Of 523 serum samples tested by hemagglutination inhibition and complement fixation, all those that were positive in complement fixation gave positive results in hemagglutination [73]. The hemagglutination test, like complement fixation, lends itself to automation.

Electron microscopy: All serum positive for HAA by immunologic tests contain virus-like particles of the type shown in Figures 2 and 3. HAA in serum can be concentrated by pelletting in an ultracentrifuge, after addition of anti-HAA to form aggregates, as shown in Figure 1. Electron microscopy, although rapid, is not a practical routine test for antigen because of its complexity and expense. It is, however, an especially useful test for detecting HAA in gradients and in

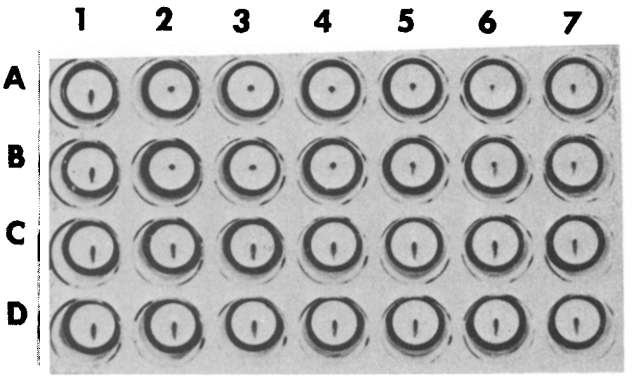

Figure 13. Patterns of hemagglutination. In testing for antibody by hemagglutination, 0.025 ml of cells coated with HAA was added to 0.025 ml of serial twofold dilutions of test serum. After incubating for one hour at room temperature, microtiter plates with V-shaped wells were centrifuged at 1,200 rpm for thirty seconds, then kept at an angle of 60 degrees for fifteen to twenty minutes before reading. Agglutinated cells appear as dots and unagglutinated cells appear as a line of red cells that streams down the bottom of the well. Rows A to D are four different serum samples being tested for antibody. Row 1 contains the highest concentration of test serum, 1:10, mixed with cells that were not coated with HAA. In rows 2 to 7, serial twofold dilutions of test serum beginning with a 1:10 dilution are mixed with cells coated with HAA. Serum in row A completely agglutinates cells up to well 6, giving an anti-HAA titer of 1:160. Serum B completely agglutinates cells through well 4, giving an anti-HAA titer of 1:40. Serum C and D contains no antibody. In order to test for antigen, a known quantity of antibody is mixed with the unknown serum. If HAA is present, antibody is neutralized and cells coated with HAA will not be agglutinated by the mixture, a so-called hemagglutination inhibition test for antigen.

TABLE IX Comparison of Tests for HAA and Anti-HAA

Technic	Approximate Sensitivity for Detecting (reciprocal titer)		Time Required for Test (hr)	Comments
	HAA	Anti-HAA		
Ouchterlony double diffusion				
Agar gel	1	1–5	24–72	Sensitivity directly proportional to number of times wells refilled or degree to which specimen concentrated by dehydration
Agarose	2	2–10	24–72	
Counterelectrophoresis	10–15	5–10	2	Can test for HAA and anti-HAA simultaneously; sensitive enough to detect most carriers of HAA; excellent for screening blood donors
Complement fixation	100–200	20–40		Excellent for screening blood donors; can be automated; unique for detecting HAA-antibody complexes
Quantitative			4	
Microtiter			2	
Complement diffusion			2	
Hemagglutination	100–200	5,000–10,000	2	Best test for anti-HAA; can be used routinely for HAA tests; can be automated
Radioimmunoassay	100–200	5,000–10,000	120–140	Too complex for routine use

purified concentrates, for HAA retains its morphologic characteristics but may lose its antigenic activity during purification procedures (Table I). Electron microscopy is also valuable in identifying circulating antigen-antibody complexes which may not be detected by immunologic tests [20,76].

Radioimmunoassay: An immunologic assay using purified HAA labeled with radioactive iodine has been described recently [75]. The test involves incubating the labeled antigen for two to three days with serum being tested for antibody, performing "chromato-electrophoresis" to separate free labeled antigen from antibody-bound labeled antigen, staining to locate the separated peaks and drying of the paper strips for counting in an automatic strip scanner. The assay for antibody takes approximately six days. In detecting antibody, the radioimmunoassay and hemagglutination assay are about equally sensitive, but hemagglutination is simpler and faster.

In detecting HAA the radioimmunoassay appears to have about the same sensitivity as complement fixation or hemagglutination but is too complicated for routine use.

Immunofluorescence: HAA was not detected by complement fixation in extracts of liver biopsy specimens from patients who had HAA-positive hepatitis [70]. However, Millman and co-workers [87] described a fluorescent antibody technic for detecting HAA in liver cells. This involves flooding smears of minced tissure preparations with rabbit anti-HAA labeled with fluorescein isothiocyanate. Fluorescent granules considered to be specific for HAA were observed within or on the nuclei of almost every liver cell in four patients with HAA-positive hepatitis but not in patients without hepatitis. The same group of workers [88] described specific HAA granules in spleen, bone marrow, testes and mesentery of some patients with HAA-positive hepatitis as well as in the liver. In addition fifteen patients who did not have HAA in their blood showed granules in the liver, a finding interpreted to indicate that the fluorescent test is more sensitive than the agar gel immunodiffusion test in detecting HAA. The fluorescent antibody technic is difficult to perform, but as Blumberg et al. [54] state, the test will prove very useful if the findings already published are supported by additional studies and confirmed in other laboratories.

Virus culture and animal transmission: Subhuman primates, especially those higher in the scale of evolution, appear to be the only animals in which HAA can be propagated, for HAA as well as anti-HAA has been found in the blood of chimpanzees, orangutans and gibbons, but not in monkeys (Table V). Although a number of preliminary reports have appeared in the past on adaptation of viral hepatitis agents to chick embryos, or tissue cultures, HAA has not as yet been propagated in tissue culture or in any of the usual laboratory animals. The goal of those attempting to develop biologic tests involving tissue culture infectivity is to approach the sensitivity of human susceptibility to infection. Only tests of this sensitivity will permit detection of the minimum amounts of virus that transmit disease and evaluation of the immune responses in terms of virus neutralization.

Table IX compares the various technics for measuring HAA and anti-HAA in blood.

Reagents. Sources of antibody and antigen: Patients given repeated transfusions of blood or blood products are the best source of high titer IgG precipi-

tating and complement fixation antibodies. About one-third of those with severe hemophilia who require frequent treatment with plasma, cryoprecipitate or fraction I have antibodies that can be utilized for diagnostic purposes [20]. The best antibodies are anamnestic or "hyperimmune" antibodies that reach peak titer about two weeks after therapy with blood or blood products that by chance contain HAA. These antibodies, which are especially suitable for complement fixation tests, arise and decay characteristically as shown in Figure 8. All antiserums have both precipitin and complement fixation properties, although different antiserums may be better for one type of test than the other. Human immunization occurs less frequently in countries such as England in which HAA carriers are less prevalent than in the United States. Less than half of the patients with hemophilia or other patients whose serums contain high titer anti-HAA have a history of hepatitis.

Excellent precipitating antibodies against HAA can be produced in animals by sensitizing them with human plasma containing HAA and absorbing the antiserum with normal plasma. Antiserums produced in this way give a line of identity with human anti-HAA [89], but these antiserums are not suitable for complement fixation procedures because the absorption step makes them markedly anticomplementary. HAA separated from plasma by double isopycnic banding in cesium chloride followed by rate zonal centrifugation in sucrose is pure enough to stimulate specific complement fixation anti-HAA antibodies in guinea pigs [19].

High quality complement fixation antibodies were found in chimpanzees that were immunized for other purposes with chimpanzee blood that inadvertently contained HAA [20,29], and similar antiserums have been produced in chimpanzees by intentional immunization with partially purified HAA [90]. Antiserums from chimpanzees are as specific as human antibodies for HAA. Immunization of higher primates could provide a good source of standard high titer complement fixation antibodies.

Specificity of reagents: Lines of identity like those shown in Figure 9 have been obtained with antigen from 300 different sources and with fifty-three different antiserums [8,20,29,70]. All antigens tested, whether from blood of patients clinically diagnosed as having "serum" or "infectious" hepatitis, fresh or stored up to twenty years at —20°C, or separated as a purified suspension of particles, gave lines of identity in agar gel diffusion and reacted similarly in complement fixation. HAA from nonhuman primates was in all respects identical to HAA from human beings [8,20,29]. Although one might expect the 200 Å structure which contains HAA to be antigenically multispecific, as yet there is no firm evidence that the hepatitis virus produces more than one antigen. Proteins of small viruses consist of multiple units of a single polypeptide, or at most several polypeptides [17], and therefore have few antigenic determinants. HAA is evidently the major antigenic determinant of the long-incubation hepatitis virus, but it would not be surprising if antiserums are found that distinguish antigenic subtypes.

There is a report by Levene and Blumberg [91] concerning an antiserum that appears to recognize a second hepatitis associated antigen. This antiserum formed early in the course of immunizing one rabbit with human plasma containing HAA and disappeared with further immunization of the same rabbit. The spur in Ouchterlony double diffusion that was considered to represent a line of nonidentity with the known HAA was too faint for photographic reproduction. This finding conceivably could reflect differences in diffusion rates of rabbit 19S and 7S antibodies of the same specificity rather than antibodies of different specificity.

Different HAA antigens no doubt will be identified in the future. For example, Zuckerman et al. [92] have found particles similar in structure to corona virus in a patient with chronic active hepatitis but have not found these particles in serum known to transmit short-incubation or long-incubation hepatitis. Also, the finding of a new antigen in stools of many patients with HAA-positive hepatitis suggests that other agents, perhaps helper viruses, may be involved in hepatitis. When additional hepatitis associated antigens are documented a system of nomenclature will be necessary.

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