(1954)] by acetylation of D-[GL-^{3}H]glucosamine (specific activity, 3.7 c/mmole) purchased from the International Chemical and Nuclear Corporation, Irvine, California.
9. A minimum of 66 percent of the radioactivity incorporated into the ghosts was recovered as glucosamine after hydrolysis with 6*N* HCl and chromatographic separation on a Dowex-50 column eluted with a gradient of from 0 to 0.05*N* HCl. Most of the fraction unaccounted for was probably also glucosamine, part destroyed during acid hydrolysis and part incompletely hydrolyzed chains retained by the column. The labeled polymers are most likely chitosan and chitin in a ratio 6.6 : 1 as determined by the relative proportion of hot 1*N* HCl soluble and insoluble radioactive material. This ratio approximates that previously found in nonlabeled walls by S. Bartnicki-Garcia and W. J. Nickerson [*Biochim. Biophys. Acta* **58**, 102 (1962)].
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Virus-like Antigen, Antibody, and Antigen-Antibody Complexes in Hepatitis Measured by Complement Fixation

Abstract. *Complement fixation techniques are described for measuring a virus-like antigen associated with viral hepatitis. Antigen was found in the blood of 98 percent of 130 patients, with the serum form of hepatitis, from whom multiple samples were obtained. Antibodies arising during hepatitis are usually combined with antigen and cause anticomplementary activity in the serum, which is reversible with excess antigen or antibody. Tests for antigen and specific anticomplementary activity can be used diagnostically and to screen blood donors for hepatitis carriers.*

Australia antigen (*1*) is present in the blood of a high proportion of patients with serum (posttransfusion) hepatitis, but it is found less frequently (*1, 2*) or not at all (*3*) in patients with infectious hepatitis. The antigen has morphologic and physical characteristics suggesting that it is hepatitis virus itself (*2, 4, 5*). Agar-gel precipitation has been the method for demonstrating this antigen and the human antibodies used have arisen in patients who had multiple exposures to blood potentially containing hepatitis virus. This report concerns measurement of Australia antigen by complement fixation (CF) techniques that are much more sensitive than agar-gel precipitation.

The antibodies we used to measure antigen were found in serums from 7 of 21 hemophiliacs, in 3 of 8 patients with refractory anemias, all but one of whom had been treated with multiple transfusions over a period of years, and in serum of 2 of 8 chimpanzees that had been transfused repeatedly with chimpanzee blood. The two chimpanzees and half of the patients who developed antibodies had no history of hepatitis. One patient developed an antibody within 10 days after his first transfusion and subsequently had anicteric hepatitis. Eleven antibodies were in the second protein peak (>60,000 and <200,000 molecular weight) of serum fractionated on Sephadex G-200 (*6*) and one was equally distributed in the first (>200,000 molecular weight) and second peaks. All of these antiserums precipitated antigen in agar gel.

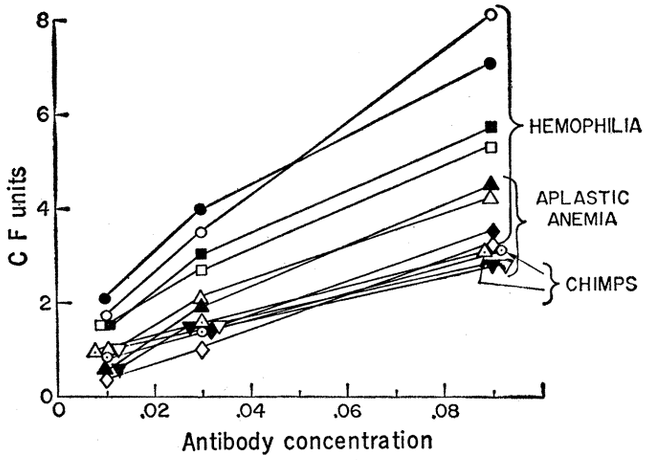
Our quantitative CF technique was developed for measuring cellular antigens (*7*). The incubation mixture (0.4 ml) contained appropriate dilutions of antigen and antibody and 10 or more hemolytic (50 percent effective) units of complement (C). Complement fixation was 85 percent complete within 15 minutes at 37°C, 95 percent complete at 30 minutes, and not significantly increased by additional incubation at 5°C. Human and guinea pig C were fixed equally. We also used a standard microtiter CF system (see *8*).

When tested at a constant concentration of antigen, the highest titered antibodies were from hemophiliacs who had received more transfusions over longer periods than had other patients or the chimpanzees (Fig. 1). Three antibodies that precipitated allotypic human beta lipoproteins in agar gel (*9*) were used as controls and did not fix C with reactive beta lipoproteins or with hepatitis antigen.

The same relative concentrations of eight different antigens produced similar CF (Fig. 2). Antigen stored as long as 15 years at −20°C or heated at 56°C for 1 hour gave results essentially the same as antigen in fresh serum. Partially purified virus-like particles, which could be separated at a density of 1.28 on cesium chloride gradients from all antigenic serums after lipid extraction (*5*), reacted in CF quantitatively like whole serum from which the particles were obtained. The similar reactions of antigens from patients with serum or infectious hepatitis or from normal gibbons, chimpanzees, and orangutans with each of 12 antibodies suggests that a single immunologic specificity is involved. The same 12 antibodies also gave agar-gel precipitation reactions of identity with these antigens, although a rabbit antiserum has been reported to differenti-

Table 1. Sensitivity of techniques for detecting hepatitis-related antigen. Aggregates of virus-like particles were prepared for electron microscopy (EM) by concentration on cesium chloride gradients (*5*). Quantitative complement fixation (CF) was performed with an amount of antibody that produced maximum fixation at each antigen dilution. In the box microtitration, 0.025-ml samples of twofold dilutions of antibody and antigen were mixed with 2 units of C in a total volume of 0.075 ml and incubated at 4°C for 18 hours before 0.025 ml of a 1 percent suspension of sensitized cells was added. Degree of complement fixation is indicated by (−) for complete hemolysis, to 4+ for no hemolysis.

Antigen dilution	Optimum antibody			CF box titration at antibody dilution				
	Agar gel	EM	CF units	30	60	120	240	480
1	3+	4+	>10	—	—	—	—	—
2	1+	3+	>10	—	—	—	—	—
4	—	2+	>10	2+	—	—	—	—
8		1+	>10	3+	1+	—	—	—
16		±	>10	4+	3+	—	—	—
32			>10	4+	4+	1+	—	—
64			>10	4+	4+	4+	±	—
128			8	4+	4+	4+	4+	—
256			4.8	4+	4+	4+	4+	—
512			2.5	4+	4+	4+	4+	±
1024			<1	4+	4+	4+	4+	±
2048			0	3+	4+	4+	2+	—
4096				±	±	—	—	—



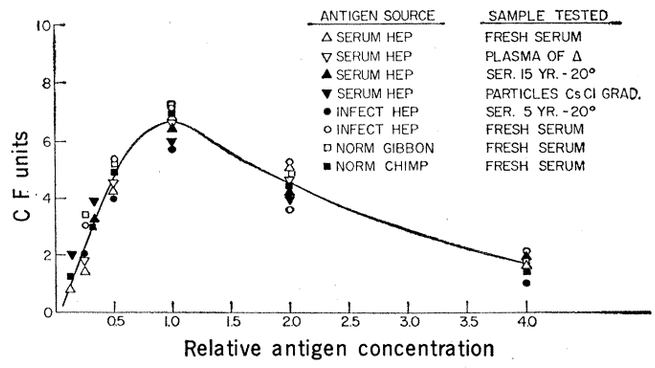


Fig. 1 (left). Complement fixation with constant antigen concentration. Antigen was serum from a patient with serum hepatitis. The concentration of antigen used, 0.005 ml per 0.4 ml of incubation mixture, fixed 10 units of C with optimum antibody concentration. All serums were inactivated for 30 minutes at 56°C. The unit of antibody concentration is milliliters of serum per 0.4 ml of mixture. Fig. 2 (right). Complement fixation with constant antibody concentration. The amount of antibody used was sufficient to fix approximately 6.5 units of C at an optimum antigen concentration, which is indicated by a concentration of 1.0 on the abscissa. The different relative antigen concentrations are based on dilutions of serums or of a saline suspension of virus-like particles. Antigen was present in 3 of 23 gibbons, 2 of 128 chimpanzees, and 3 of 19 orangutans.

ate two hepatitis antigens in agar gel (*10*).

Of the techniques for detecting antigen shown in Table 1, electron microscopic identification of aggregated virus-like particles (*5*) was fourfold, quantitative CF 200- to 300-fold, and microtiter CF 1000- to 2000-fold more sensitive than agar-gel precipitation. Most hepatitis serums that are positive in agar-gel precipitation have an antigenic titer similar to the example shown in Table 1. The antigen with the highest titer in serum from a patient with hepatitis was detected by agar-gel precipitation at a dilution of 1:8; electron microscopy, 1:64; by quantitative CF, 1:2048; and by microtiter CF, 1:16,384. The lowest titered antigenic serums were detected only by the microtiter system.

Table 2 compares the incidence of antigen in the blood by agar-gel precipitation and CF techniques in patients with serum and infectious hepatitis. Measurement of antigen by CF in serum hepatitis appears to be a reliable diagnostic test, provided that several weekly samples are obtained shortly after onset of clinical symptoms, for antigen in the blood always appeared at some time after symptoms, although it also was frequently detected several weeks to several months before symptoms and after recovery (*2, 11*). Although cases in groups 1 and 2 could not be related to transfusion or inoculation, conceivably infection could have been acquired by some obscure parenteral route. Whether clinical differences between serum and infectious hepatitis are due to portal of entry or differences in virus per se remains an open question. The virus measured in our study may be only one of several virus groups responsible for hepatitis.

Previous attempts to demonstrate antibodies specific for hepatitis, with acute and convalescent serums, have been unsuccessful (*12*). A clue to the problem was finding that 95 percent of patients shown in Table 2, group 4, developed anticomplementary (AC) activity in their serums at some time during the course of hepatitis; that is, their serum alone fixed C. Serum dilutions that fixed two or more units of C varied from 1:4 to 1:>512. Anticomplementary activity almost always precedes, but could coexist with, appear after, or occur without antigen in the blood being detectable by addition of antibody (*11*). The temporal relation between AC activity and antigen in the blood of the type shown in Table 3 occurred sufficiently often to suggest that circulating antigen-antibody complexes were responsible for AC activity. Anticomplementary activity occurring characteristically 4 to 6 weeks after exposure could be due to antigen-antibody complexes, for the exceptional cases without AC activity at this time had free antigen in their serums. Decreases in AC activity when the blood antigen reached its greatest concentration, weeks 12 to 19, were consistent with effects on CF of high ratios of antigen to antibody (Fig. 2); and return of AC activity during convalescence possibly reflected a decreased ratio. On Sephadex G-200 fractionation, serum AC activity appeared chiefly in the first peak—the same peak

Table 2. Incidence of antigenemia in hepatitis by agar-gel precipitation and CF techniques. The four groups of patients are: group 1, soldiers involved in a probable common source outbreak of infectious hepatitis at Ft. Belvoir, Virginia, on whom one or two samples were available when liver function tests first became abnormal; group 2, cases of endemic hepatitis from Ghana, on whom occasional samples were taken during acute and convalescent phases; group 3, patients with posttransfusion hepatitis, from whom serums were collected once or twice during the acute and convalescent p'ases of the disease; group 4, volunteers exposed parenterally in a 1952–54 study of a variety of blood products known to transmit viral hepatitis and on whom blood samples were collected at weekly intervals after exposure (*14*). Normal controls were healthy hospital employees (group 5). In the quantitative screening procedure, an amount of antibody sufficient to fix 8 units of complement with optimum antigen concentration was incubated with hepatitis serum at dilutions of 1:4, 1:40, and 1:200 final dilutions. In the microtiter CF, screening was done with one concentration of antibody and twofold serial final dilutions of hepatitis serum from 1:16 to 1:2048.

Group	Individuals (No.)	Total samples	Individuals with antigen (%)	
			Agar gel	CF
1	8 icteric	16	0	100
	29 anicteric	45	7	45
2	112	296	16	59
3	24	38	8	54
4	130	4000	62	98
5	242	242	0	0

Table 3. Relation between antigen in the bloodstream and anticomplementary activity. Serum samples were from a patient who developed hepatitis after a transfusion. Samples were obtained at weekly intervals. Antigen was measured by agar-gel precipitation and by quantitative CF. The CF titer is the reciprocal maximum dilution of serum that fixed more than 2 units of C in the presence of optimum antibody. The patient's serum alone at 1:20 dilution fixed amounts of C listed as anticomplementary (AC) activity. The amount of antigen added to reverse AC activity was approximately 15 times the amount of optimum antigen for an amount of antibody capable of fixing 8 units of C at an optimum ratio of antigen to antibody (that is, sufficient antigen to cause less than 1 unit of CF as shown in Fig. 2). The source of antigen was highly antigenic serum (titer, 1 : > 10,000 by microtiter CF) from patients with serum or infectious hepatitis, or from nonhuman primates, as well as antigen purified on CsCl gradients. The amount of antibody added to reverse AC activity was 0.15 ml of the antibody with the highest titer (shown in Fig. 1), per 0.4 ml of mixture. Antigen or antibody was added to AC serum and incubated for 2 hours at 37°C or for 4 hours at 24°C before C was added.

Time after exposure (weeks)	Serum antigen + antibody		Serum AC activity (units)			
			Serum alone	Serum + excess antigen		Serum + excess antibody
	Agar gel	CF titer		Incubated before CF	No inc. before CF	Incubated before CF
3	0	0	0	0	0	0
4	0	0	6.0	0	4.5	1.5
6	0	0	7.5	0	6.5	2.8
8	0	40	2.1	0	1.8	
12	+	640	0	0	0	
16	+	320	1.5	0	0	
19	0	160	1.8	0	1.5	
21	0	0	7.5	0	6.5	2.5
28	0	0	7.4	0	7.0	3.2
32	0	0	4.9	0	3.0	2.0
36	0	0	1.5	0	1.0	0

that contains virus-like particles of antigenic serums. Three high-titered AC serums that were examined by electron microscopy after sedimentation on CsCl gradients, contained virus-like particles which were anticomplementary. These particles appeared to have an outer coat about 2 nm thick, which could be removed by extraction with dichlorodifluoromethane (*5*). This fluorocarbon removes antibody from neutralized virus, with the virus being in the aqueous phase and precipitated antibody in the organic phase (*13*). Extraction of AC serum for 2 hours at room temperature by Vortex mixing with an equal volume of fluorocarbon decreased or eliminated AC activity and often doubled the antigenic activity detectable with added antibody. Thus, hepatitis virus in complex with antibody appears to be responsible for AC activity.

Further evidence that antigen-antibody complexes account for AC activity is the ability of excess antigen to reverse it. Reversal was a time-dependent reaction, the rate being inversely proportional to the amount of antigen added. For example (Table 3), amounts of antigen that completely eliminated AC activity when incubated with AC serum for approximately 2 hours at 37°C or 4 hours at 24°C before addition of C, only slightly reversed AC activity if added immediately before C. Most antibodies used in Fig. 1 showed a "prozone" phenomenon of inhibiting CF at high ratios of antibody to antigen; and these antibodies in high concentration reversed AC activity as did excess antigen. Serums from normal individuals and from hepatitis patients without demonstrable antigen or antibodies in the blood did not reverse AC activity. Free antibody was detected much less frequently than AC activity. Complement fixing antibody could be demonstrated in only 3 of 22 hepatitis cases on whom weekly serum samples were obtained during the month after disappearance of antigen in the blood. None of the patients developed antibodies that precipitated antigen in agar gel (*2*). Thus it appears that CF antibody in most cases of hepatitis circulates while bound to antigen and manifests its presence as AC activity. Conversely, AC activity reversible with specific antigen or antibody appears to be as indicative of viremia as measurement of free antigen.

Complement-fixation techniques provide a valuable diagnostic test for hepatitis and lend themselves to screening large numbers of blood donors to exclude carriers of hepatitis virus. Screening tests must include procedures for detecting not only free antigen, but also the AC activity of antigen-antibody complexes that can be reversed with specific antigen or antibody. Antigen in the blood may be confirmed by electron microscopy (*5*).

N. RAPHAEL SHULMAN
Clinical Hematology Branch, National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland 20014
LEWELLYS F. BARKER
Laboratory of Viral Immunology, Division of Biological Standards, National Institutes of Health

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Radiation Leukemia Virus: Quantitative Tissue Culture Assay

Abstract. *Radiation leukemia virus does not propagate in tissue cultures from either Swiss or C57BL mouse embryos, but it does augment focus formation by the defective Moloney leukemia pseudotype of murine sarcoma virus in Swiss mouse cells and thus can be quantitatively assayed.*

X-irradiation of C57BL mice, which have a low spontaneous incidence of leukemia, induces lymphomas from which a leukemogenic virus (Rad LV) and viral-specific antigen can be consistently recovered (*1*). The host range, the ensuing pathology, and the thymic