Comparison of three serological methods for the detection of hepatitis-associated antigen

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SYNOPSIS Serum hepatitis (Australia) antigen in the sera of hepatitis patients and carriers can be detected in one and a half to three hours by crossover electrophoresis. The method is more sensitive than the immunodiffusion technique commonly employed in this field. It is of the same order of sensitivity as complement fixation but is less complicated.

Crossover electrophoresis is thus the method of choice for the rapid screening of sera for hepatitis antigen; complement fixation may be used for quantitative determination of antigen in positive cases.

Immunodiffusion and complement-fixation tests have been used for the detection of a specific antigen which appears in the blood late in the incubation period and early in the acute phase of serum hepatitis. It is called variously Australia antigen (Blumberg, Gerstley, Hungerford, London, and Sutnick, 1967), SH antigen (Prince, 1968), and hepatitis antigen (Gocke and Kavey, 1969); electron microscopy shows that it is constantly associated with the presence in the serum of spherical particles about 20 nm in diameter. Larger particles have been described by Dane, Cameron, and Briggs (1970). These are approximately 42 nm in diameter and consist of antigen-coated structures resembling virus particles. The outer layer may be the source of the 20 nm particles and the characteristic tubular forms of similar width that are sometimes seen.

In both immunodiffusion and complement-fixation tests the source of detector antibody is the serum of certain patients, usually haemophiliacs, who have received large numbers of transfusions and, presumably, repeated exposures to antigen. Most workers carrying out immunodiffusion tests have followed Prince in using a micro-Ouchterlony-Elek technique to detect lines of precipitation developed by double-diffusion in an agarose gel. Those who have used the complement-fixation test have found it to be more sensitive than the immunodiffusion test for the detection of antigen in serial dilutions of serum (Purcell, Holland, Walsh, Wong, Morrow, and Chanock, 1969; Shulman and Barker, 1969) and more frequently positive in patients with hepatitis (Cossart and Vahrman, 1970).

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Positive tests for antigen have been obtained in persons incriminated on epidemiological grounds as carriers of serum hepatitis virus (Gocke, Greenberg, and Kavey, 1969; Turner and White, 1969; Zuckerman and Taylor, 1969) and it is clear that a simple but sensitive test for antigen may be the key to controlling hepatitis associated with techniques which require blood transfusions, including major surgery and haemodialysis. The complement-fixation test, although the more sensitive of the two tests most commonly used, is time consuming and involves difficulties of interpretation when sera are anticomplementary (Gocke, Greenberg, and Kavey, 1970). Prozone effects, too, are not uncommon and in consequence relatively large amounts of detector serum must be used. These are serious disadvantages if, for example, mass screening of blood donors is contemplated.

For the detection of small amounts of antigen in forensic practice, Culliford (1964) used crossover or counter-electrophoresis and this technique has also been tried as a rapid and sensitive test for SH (Australia) antigen (Pesendorfer, Krassnitzky, and Wewalka, 1970; Gocke and Howe, 1970; Prince and Burke, 1970). We have been using this method for the investigation of patients with suspected serum hepatitis and have compared our findings with the results of immunodiffusion and complement-fixation tests carried out in parallel. The subjects studied were cases of post-transfusion hepatitis, drug addicts, and hospital staff and relatives who developed the disease while caring for patients on maintenance haemodialysis.

Our findings indicate that crossover electro-

phoresis is as sensitive as complement fixation for the detection of SH antigen but takes only a few hours to perform and problems associated with prozone and anticomplementary effects are eliminated.

Methods

IMMUNODIFFUSION

For the detection of serum hepatitis antigen, a micro-Ouchterlony-Elek technique was used to demonstrate lines of precipitation developed by double-diffusion in an agarose gel (Prince, 1968; Turner and White, 1969). The gel consisted of 0.9% agarose (B.D.H.) dissolved in a buffer consisting of 0.1M sodium chloride, 0.01M tri (hydroxymethyl) methylamine (Tris), and 0.001M ethylenediamine tetraacetic acid (EDTA) containing 1 mg per ml protamine sulphate. The detector antiserum was from a haemophilic patient who had received more than 9,000 units of blood or blood products.

Preliminary tests showed that lines of precipitation were sharper and that weak reactions were more clearly defined when the antiserum was extracted with acetone and ether (Havens, 1958) and the resultant powder reconstituted in distilled water to one-quarter of the original serum volume. Further improvement in clarity was obtained by initial incubation of the tests at 37°C; some sera which were negative when tested at room temperature gave weak positive reactions after incubation at 37°C for 18 hours. All immunodiffusion results reported here relate to tests with concentrated antiserum which were read after incubation in a humid atmosphere at 37°C for 18 hours and subsequently at room temperature for two days.

COMPLEMENT FIXATION

For these tests a four-volume microtechnique was used with Takatsky pipettes which delivered 0.025 ml volumes of the test serum in doubling dilutions from 1/2 to 1/256, antiserum (unconcentrated) at optimal dilution, complement (2 units), and sensitized sheep erythrocytes (2% suspension). All sera were inactivated at 56°C for 30 minutes. Fixation of complement took place at 4°C overnight.

The optimal dilution of antiserum was determined by the chequerboard method with, as a source of antigen, serum obtained from a patient just before the onset of clinical hepatitis when by electron microscopy tubular and spherical forms of antigen without clumping were seen. An antigen-positive serum with a complement-fixation titre determined by tests with several different antisera by Dr Y. E. Cossart reacted to titre with our antiserum at optimal dilution.

CROSSOVER ELECTROPHORESIS

The method used was based on that of Culliford (1964). Glass slides, 7.6 cm × 2.5 cm, or Hyland 'immunoplates' of similar size were coated with 3.0 ml of agarose gel as used in the immunodiffusion test. Twenty-four wells, in three columns of four pairs, were cut in the gel on each slide. The axis of each pair of wells was parallel to the electrophoretic axis along the length of the slide. Paired wells were 2 mm apart; each was of 2 mm diameter and of 2 µl capacity.

Sera under test for antigen were placed in the cathodal and antiserum in the anodal wells. Antiserum was either untreated or concentrated by acetone-ether extraction as in the immunodiffusion test. The most consistent results were obtained with concentrated antiserum but there is clearly a need to standardize antisera which vary greatly in potency. In the tests reported here the activity of antisera was assessed in relation to strong and weak antigen-positive control sera included in each group of 12 tests. Slides were connected to the buffer compartments of the electrophoresis apparatus by Whatman 3MM paper wicks. The electrode vessel buffer, pH 8.4, consisted of 0.05M barbitone and sodium barbitone. A Vokam power pack was adjusted to deliver a constant current of 4 mA per slide for a period of three hours. Under these conditions the water-cooling system necessary in high-voltage electrophoresis (Prince and Burke, 1970) was not required.

Moderate or weak reactions appeared after two to three hours as straight lines of precipitate midway between the antigen and antibody wells. Strong reactions were clearly visible in less than two hours; subsequently continued passage of current and further migration of antigen led to blurring of the sharp lines and their replacement by arcs closely applied to the antiserum wells (Figure).

Results

ACUTE PHASE OF SERUM HEPATITIS

Specimens of serum, taken within three weeks of the onset of the disease, were available from 28 patients. These included eight who had developed hepatitis 60 or more days after blood transfusions; five, of whom three were drug addicts, with hepatitis clearly related to injections; and 15 with haemodialysis-associated hepatitis, 11 of whom were staff of a haemodialysis unit and four relatives of patients on home dialysis.

Table I shows the results of immunodiffusion, crossover electrophoresis, and complement-fixation tests on these sera. Both immunodiffusion and crossover electrophoresis tests were positive in 18 (64%) of the 28 sera; five more were positive by the

Number of Patients	Positive Precipitation Reactions by		Complement-fixation Titres							
	Immunodiffusion	Crossover Electrophoresis	<2 or Inconclusive	2	4	8	16	32	64	128 or More
18	18	18	1	0	0	0	2	2	3(1)	10 (1)
5	0	5	0	1	1	3	0	0	0	0
5	0	0	5	0	0	0	0	0	0	0

Table I Tests for SH (Australia) antigen by immunodiffusion, crossover electrophoresis, and complement fixation in 28 patients with suspected serum hepatitis¹

¹Figures in parentheses represent sera which were anticomplementary at 1/4 or more but for which titres were at least four-fold greater than the anticomplementary levels; those in which the difference was less than four-fold were classed as inconclusive.

crossover electrophoresis test. Of 23 sera positive by crossover electrophoresis, 22 had titres by the complement-fixation test of 1/2 or more and one was anticomplementary. Complement-fixation titres were within the range 1/8 to 1/128 or more in sera positive by both immunodiffusion and crossover electrophoresis, and 1/2 to 1/16 in those positive by crossover electrophoresis alone. The five sera negative by both immunodiffusion and crossover electrophoresis were negative or anticomplementary in the complement fixation test.

Table II shows the distribution of positive results in the three groups of patients. In the largest, the haemodialysis-associated group, the number of those giving positive reactions was increased from 11 to 14 by the use of crossover electrophoresis or complement-fixation tests in addition to immunodiffusion. In the group of eight cases of post-transfusion hepatitis the crossover electrophoresis test gave the best results but detected positive reactions in only half the patients.

The one patient in the haemodialysis-associated series who was negative by all three tests was a nurse who pricked her finger while taking blood from an antigen-positive patient. Eleven weeks later, although not ill, she was found to have a marked rise in serum glutamic pyruvic transaminase but the serum bilirubin level was normal.

Type of Case	Total Cases	No. of Cases Positive by		
		Immunodiffusion	Crossover Electrophoresis	Complement Fixation
After transfusion	8	3	4	3
Injection related	5	4	5	5
Haemodialysis associated	15	11	14	14
Total	28	18	23	22

Table II Positive tests for serum hepatitis (Australia) antigen in serum hepatitis

WEAKENING OF POSITIVE REACTIONS IN CONVALESCENCE

Serial serum samples were available from nine antigen-positive patients in the haemodialysis-associated hepatitis group. Specimens had been collected after the onset of the disease at weekly or greater intervals over periods of several months.

Table III shows that during convalescence there was a steady weakening of positive reactions detected by immunodiffusion and crossover electrophoresis tests and a parallel decline in complement-fixation titres. At the seventh or eighth weeks after onset samples were available from five patients, all of whom were negative by the immunodiffusion test but positive at 1/2 or 1/4 by the complement-fixation test; four were positive by crossover electrophoresis.

There was great variation in the duration of positive reactions; but in eight of the nine cases positive crossover electrophoresis reactions were obtained after the immunodiffusion test had become negative. The crossover electrophoresis test was positive in two cases until the 11th or 12th week after onset and in one instance this method detected

Fig. Crossover electrophoresis tests in 10 serum samples taken from one patient at intervals during recovery from serum hepatitis. In the left-hand and centre columns of wells are the first eight samples, all positive, the last taken 11 weeks after onset. In the right-hand column are the remaining two samples, both negative, and positive and negative controls.

By immunodiffusion, the first five samples only were positive, the last taken seven weeks after onset.

Patient	Method	Results of Three Tests on Serum Specimen								
		Before Onset	Weeks after Onset							
			1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16
1	ID COE CF	— + 8		(+) + 8	— + AC	— — 2				
2	ID COE CF	+ + 128		+ + NT		— (+) 2	— — 2			
3	ID COE CF		+ + 128		— — AC					
4	ID COE CF		+ + ≥256		(+) + 8		— + 2	— (+) 2	— (+) 2	— — <2
5	ID COE CF			+ + ≥256	+ + 4	— (+) 2	— — 2			
6	ID COE CF		+ + ≥256		+ + 16	+ + 2	— + 2	— (+) AC	— — <2	
7	ID COE CF		+ + !28	(+) + 8	— + 4	— + 4		— (+) <2	— — 2	
8	ID COE CF		— + 8	— — <2						
9	ID COE CF		+ + 64	(+) + 16	— + NT		— — <2			

Table III *Weakening of positive reactions for serum hepatitis (Australia) antigen during convalescence*

ID = immunodiffusion COE = crossover electrophoresis CF = complement fixation

+ = positive, (+) = weakly positive, — = negative

AC = anticomplementary NT = not tested

antigen as late as the 13th week. With few exceptions there was close correspondence between weakening and loss of crossover electrophoresis reactions and the parallel decline in complement-fixation titres. With those sera which gave the weakest crossover electrophoresis precipitin lines, complement-fixation tests were either positive only at 1/2 or, in one case, negative; with four sera, however, with complement-fixation titres of 1/2, a precipitin line could not be demonstrated by crossover electrophoresis. Sera obtained before the onset of illness were available from two patients. From one of these (patient 1) serum collected two weeks before onset was negative by immunodiffusion but positive by crossover electrophoresis and complement-fixation.

CHRONIC CARRIERS OF ANTIGEN IN A HAEMODIALYSIS UNIT

Immunodiffusion tests for serum hepatitis antigen

become strongly positive when serum hepatitis develops in patients with chronic renal failure who are on maintenance haemodialysis; these reactions do not weaken significantly with the passage of time (Turner and White, 1969). Sera from five of these patients, all with complement-fixation titres of 1/256 or more, were tested by the crossover electrophoresis technique; in each case, a typical strong reaction was obtained with the development of a precipitin line in less than two hours. Sera from five patients from the same unit who had been consistently negative by immunodiffusion were also negative by the crossover electrophoresis and complement-fixation tests.

We tested five sera taken at about yearly intervals from a patient on haemodialysis who developed hepatitis in 1966; all these samples, including that taken in 1970, were strongly positive by immunodiffusion and crossover electrophoresis tests and had complement-fixation titres of 1/256 or more.

Discussion

Our findings confirmed those of other workers that complement fixation is more sensitive than immunodiffusion for the detection of serum hepatitis (Australia) antigen. This superiority was maintained despite improvements in the double-diffusion technique by the use of concentrated antiserum and preliminary incubation at 37°C.

Gocke and Howe (1970) showed that crossover or counter electrophoresis was at least 10 times more sensitive than conventional immunodiffusion methods for the detection of Australia antigen in serial dilutions of serum. Crossover electrophoresis makes use of the fact that during electrophoresis in a negatively charged gel endosmotic flow causes the slow gamma globulins, including antibody, to be displaced towards the cathode, ie, in a direction opposite to that of the faster antigen. By appropriate arrangement of wells, antibody and antigen can be made to converge and the loss of these reactants by diffusion in unwanted directions in simple double-diffusion is avoided. Precipitin lines develop earlier and in our tests reached maximum intensity in one and a half to three hours, depending on the strength of the reaction, despite the fact that endosmosis is relatively weak in agarose gel.

In the investigation of 28 patients with hepatitis related to blood transfusion, injection, or haemodialysis we found that for the detection of serum hepatitis antigen crossover electrophoresis was of the same order of sensitivity as complement fixation. In the acute phase of hepatitis, more antigen-positive patients were discovered by crossover electrophoresis than by immunodiffusion. Those positive by crossover electrophoresis only were the patients with the lowest complement-fixation titres.

Our study of nine antigen-positive patients during convalescence confirmed that cross-over electrophoresis detected positive reactions in sera negative by immunodiffusion. In each case there was weakening and finally loss of positive reactions by all three tests but precipitin lines could be demonstrated by crossover electrophoresis after the immunodiffusion test had become negative; there was a close parallel between positive reactions detected by crossover electrophoresis and complement-fixation titres of 1/2 or greater.

Although the crossover electrophoresis and complement-fixation tests are thus of comparable sensitivity, the complement-fixation method has certain disadvantages. It is time-consuming to perform and overnight fixation of complement is necessary. Because of prozone effects titration of each test serum over a range of dilutions is essential.

Difficulties in interpretation may arise with anti-complementary sera. By contrast the crossover electrophoresis test is less complicated and can be completed within three hours.

It is clear that the crossover electrophoresis and complement-fixation tests are the methods of choice for the detection of serum hepatitis antigen in cases of hepatitis. We have found that a convenient combination of the two tests is to screen sera initially by crossover electrophoresis and then to use the complement-fixation method for the quantitative determination of antigen in positive cases.

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