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Serodiagnosis of viral hepatitis A: Rise in antibody titre and evaluation of three methods for detecting early and late antibodies

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Summary. A serological investigation was made on patients with viral hepatitis A and individuals with a past history of this disease. Titration of antibody in sequential samples was found to be of no help in diagnosis. Separation of early (IgM) from late (IgG) antibodies by protein A or by 2-mercaptoethanol did not prove to be convenient for the serodiagnosis. A chromatographic separation of late and early antibody was found to be satisfactory, and equivalent to a radioimmunoassay for IgM-antibodies.

Since hepatitis A virus (HAV) was discovered by Feinstone et al.[2], various methods have been described for the detection of anti-HAV antibodies: for instance, immune electron microscopy[2], immune adherence haemagglutination[3,4], complement fixation[5], radioimmunoassay[6,7], and more recently the enzyme-linked-immuno-sorbent assay[8,9].

Anti-HAV antibodies persist indefinitely. In our region, they were shown to be detectable in the serum of about half the normal adult population[10]. For diagnostic purposes, it became necessary to distinguish such late antibodies (IgG) from those produced at the onset of disease (IgM). Therefore, in addition to measuring the rise in titre between 2 early serum samples, several authors have used methods for the specific detection of IgM. These included: 1. inactivation of IgM with 2-mercaptoethanol (2-ME)[11], 2. removal of IgG by binding to staphylococci[12], 3. separation of immunoglobulin classes by ultracentrifugation[13], and 4. detection of IgM alone using anti-IgM specific antibody[9,14].

The aim of the present study was to compare 4 different procedures used to assess the acute phase nature of anti-HAV antibody production: 1. Serial samples of serum taken after onset of disease were titrated in a commercial radioimmunoassay (RIA) to look for a possible rise in titre. To distinguish IgM from IgG activity, samples were assayed in the same RIA, 2. before and after inactivating IgM with 2-ME, 3. before and after complexing IgG with staphylococcal protein A, and 4. before and after separating IgM by chromatography.

Materials and methods. 14 patients (6 ♂, 8 ♀) with acute viral hepatitis demonstrated by clinical evidence and rise in transaminase levels were studied. The markers of hepatitis B virus infection were absent in all cases. Sera were obtained at the onset of the disease and at weekly intervals until the 2nd or 3rd month. Sera taken after 2 months were considered as 'late' samples, sera taken before day 15 as 'early' samples. Seven healthy individuals with anti-HAV antibody and a past history of acute hepatitis A, 6 months to 5 years previously, also provided serum samples for the study of late antibodies.

Titration of anti-HAV antibody. A commercial RIA was used throughout (HAVAB™ kit, Abbott GmbH, Diagnostics Division, FRG). Its principle is a competition between the patient's antibody (any class of immunoglobulin) and I^{125}-labeled-antibody for a solid phase-bound antigen. Titration was performed by diluting the samples from $^1/_2$ to $^1/_{3200}$ in PBS. The measurements were made in duplicate on sequential serum samples. The titre was the last dilution below a cutoff value, calculated from the values of a positive and a negative control.

IgM inactivation by 2-mercaptoethanol (2-ME). 200 µl of

Table 1. Effect of protein A treatment, 2-ME treatment and chromatographic separation on the anti-HAV titre of serum from 7 healthy individuals who had recovered from acute hepatitis A infection

Case No.	Titre of anti-HAV antibody			
	Nontreated	After 2-ME treatment	After protein A treatment	After chromatographic separation (IgM fraction)
1	100*	100	20	neg.
2	1600	1600	200	neg.
3	200	200	50	neg.
4	100	100	20	neg.
5	1600	1600	400	neg.
6	100	100	20	neg.
7	20	20	5	neg.

*Reciprocal dilution.

Table 2. Anti-HAV titres in acute and late phase sera from 14 cases of acute hepatits A, showing the effect of 2-ME treatment

Patient No.	Titre of anti-HAV antibody 'Early' sera (< 15 days) Nontreated	After 2-ME treatment	'Late' sera (> 60 days) Nontreated	After 2-ME treatment
1	20*	5	3200	3200
2	400	100	1600	3200
3	200	100	ND	ND
4	100	20	ND	ND
5	100	20	ND	ND
6	200	50	400	400
7	200	20	400	400
8	200	50	800	800
9	400	100	800	800
10	100	20	800	800
11	100	10	800	400
12	100	20	100	50**
13	200	50	ND	ND
14	200	50	ND	ND

*Reciprocal dilution; **serum after 45 days.

undiluted serum were incubated with the same volume of 0.2 M 2-ME for 15 min at 37 °C. The preparation was then dialyzed for 36 h against PBS, and submitted to anti-HAV titration.

Binding of IgG with protein A. 200 µl of serum were diluted 1:20 in PBS. 50 mg protein A-sepharose CL-48 (Pharmacia, Uppsala, Sweden) were added to the diluted serum for 30 min at room temperature and centrifuged for 10 min at 2000 × g. The supernatant was titrated as before.

IgM/IgG separation. The procedure described by Pyndiah et al.[15] was used. Briefly, 100 µl of serum were introduced into a small column containing 3 ml of Biogel A-5 m (BioRad Laboratories GmbH, Munich), simultaneously with 5 µl of dextran blue PM 2.10^6 (concentration 50 mg/ml) in order to label the IgM containing fractions. The fractions were then eluted with PBS pH 7.4, filtered on a micropore filter and tested for the presence of anti-HAV.

Finally, the results were compared with those of a commercial radio-immunoassay, which bad become available in the meantime for the direct detection of antibodies of the IgM class (HAVAB-M, Abbott Laboratories GmbH, Diagnostics Divison).

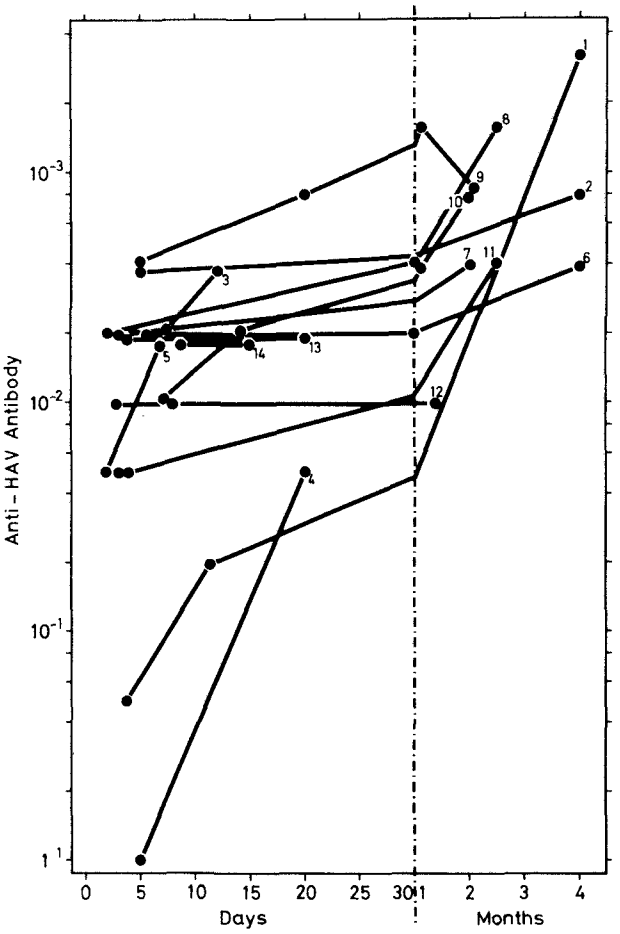
Results. The rises in anti-HAV titre after onset of disease are shown in the figure. In all individuals but two, the titre was already elevated in the first serum collected. A clear increase (more than 2 dilutions) between the 1st and the 2nd sample (at an interval longer than 2 weeks) was demonstrable in only 4 out of 9 cases. Table 1 shows the results obtained when serum samples from 7 individuals with a past history of hepatitis A were pretreated simultaneously with 2-ME, protein A and chromatography. 2-ME did not decrease the titres, whereas the concentration of the antibody was notably decreased after protein A treatment. No anti-HAV activity was found in the IgM fraction yielded by the chromatographic separation.

2-ME treatment decreased the titre of anti-HAV in samples taken early in the acute disease by at least 2 dilutions in 13 out of 14 cases (table 2). In the remaining case (No. 3), the

Table 3. Presence of anti-HAV antibody in the IgM fraction of early sera separated by chromatography, and demonstration of this antibody by HAVAB-M RIA. ND = not done

Patient No.	Anti-HAV antibody in early sera (< 15 days) IgM fraction obtained by chromatographic separation	HAVAB-M RIA Count per minute
1	+	8220*
2	+	11760
3	+	ND
4	+	3850
5	+	ND
6	+	11350
7	+	ND
8	+	8200
9	+	10710
10	+	12240
11	+	ND
12	+	7930
13	+	7850
14	+	8380

*Mean of negative controls: 605. Cutoff value 1790. Results above this value were considered positive in this test.



Titration of anti-HAV antibody during the course of acute hepatitis A.

Table 4. Effect of treatment of acute hepatitis A sera with protein A, showing comparison between acute phase sera (1st and 2nd column) and late phase sera (3rd and 4th column). ND = not done

Patient No.	Titre of anti-HAV antibody 'Early' sera (< 15 days)		'Late' sera (> 60 days)	
	Non-treated	After protein A treatment	Nontreated	After protein A treatment
1	20*	10	3200	1600
2	400	200	1600	400
3	200	200	ND	ND
4	100	50	ND	ND
5	100	50	ND	ND
6	200	200	400	100
7	50	50	400	100
8	200	100	1600	400
9	400	400	400	100
10	100	50	400	200
11	100	100	400	100
12	50	50	100	50
13	100	100	ND	ND
14	400	200	ND	ND

*Reciprocal dilution.

decrease was of 1 dilution only: $^1/_{200}$ before and $^1/_{100}$ after treatment. In the corresponding late sera, which could be obtained from 9 patients, the antibody activity was unchanged by 2-ME in all but one case (difference of 1 dilution). The IgM fractions obtained by chromatography of the early serum samples were positive for anti-HAV by RIA in all 14 cases. These results were all confirmed by the RIA detecting IgM antibodies (table 3). The effect of pretreatment by protein A of early samples from the same group of patients with acute hepatitis is shown in table 4. In no case were the titres of anti-HAV decreased by more than 1 dilution: in 7, the titres remained identical and, in 7 others, they decreased by 1 dilution. In the 9 late serum samples, the concentration of anti-HAV decreased by 2 dilutions in 6 and by 1 dilution in 3.

Discussion. It is evident that patients responded very differently from each other in their production of anti-HAV after onset of acute hepatitis A (fig.). Several had already acquired a high concentration of the antibody when the 1st serum sample was taken, which was not increased further during the period of illness. In our cases, a firm serological diagnosis of recent hepatitis A, based on titrations of samples taken at intervals longer than 2 weeks, could only have been made in 4 of 9 cases. Similar results have already been described: Frösner[16] did not find a negative titre in any of the 1st serum samples of acute hepatitis A studied. Norkraus et al.[17] could base the diagnosis of acute hepatitis A on an increase in antibody titre in only 16 of their 40 cases; they diagnosed the remaining cases by using a method detecting IgM anti-HAV. In addition, titration necessitates the repetition of RIA measurements on several dilutions of serum, which considerably increases the cost of the procedure. A RIA detecting total anti-HAV antibody (IgG and IgM) is thus of no practical use in the serological diagnosis of recent hepatitis A, unless used in conjunction with techniques allowing the separation of IgM from IgG.

In the present study, serum treatment by 2-ME seemed to be a valuable adjunct to anti-HAV titration, since it distinguished in all cases between acute phase sera and those taken later than the 60th day of disease. The absence of inhibition of 2-ME on the 7 S IgM did not seem to play a role in this model. This procedure also has the advantage of allowing serological diagnosis on a single early serum sample, but is not economical since 2 titrations of the sample are needed, each requiring at least 3-4 runs in the RIA. The smell of 2-ME is also an important drawback.

Absorption of IgG antibody by protein A seemed to be of less value in our study, since its effect on antibody titre was not clearly different in initial and late serum samples. The absence of effect of protein A on subclass 3 of human IgG could perhaps account for these results[18]. It is also possible that an early IgG response and the persistence of some IgM anti-HAV[13] might explain the poor results obtained by the 2-ME and protein A methods.

The chromatographic separation of IgM antibody, followed by RIA determination of anti-HAV activity on this fraction, best allowed the identification of acute phase antibody. The IgM fraction was positive for anti-HAV antibody in the 14 acute phase sera and negative in the 7 individuals with a past history of hepatitis A. This method does not require any expensive titration. It could replace expensive radioimmunoassays for direct IgM-antibody determinations, with which it is qualitatively equivalent.

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