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Diagnosis of recent hepatitis A infection: a comparison of two methods for detecting specific IgM

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SUMMARY

Radioimmunoassay (RIA) tests for anti-hepatitis A virus (HAV) IgM were carried out on 728 sera: 382 were tested by both a method using an anti-μ serum bound to a solid phase and a method involving preliminary separation of IgM by sucrose density gradient (SDG) centrifugation, 354 by the anti-μ method alone and two by the SDG method alone. Similar proportions of sera were found to be positive by each method (42·5%, 41·7%), but equivocal results were commoner by the SDG method (4·7% compared with 1·5%). There were 21 (5·5%) discrepant results from the sera tested by both methods, 20 of which could have been due to the higher sensitivity of the anti-μ method.

The SDG method generally gave unequivocal results on sera collected within six weeks of the onset of jaundice. Separation of the IgM fraction by re-orientation centrifugation was quick, but otherwise offered no special advantage over separation on a swing-out rotor. The use of 2 mercaptoethanol (2 ME) reduction to assess the purity of the IgM fraction increased confidence in the specificity of the test. It led, however, to the exclusion of 16 reactive sera (4·2%), all of which were found to be positive in the anti-μ test.

The anti-μ method gave better discrimination between positive and negative sera than the SDG method and detected IgM both earlier and later in infection. The results of tests designed to check the specificity of the anti-μ procedure were satisfactory. As it is potentially cheaper and easier to perform, the anti-μ method seems, in all respects, to be superior to the SDG method.

INTRODUCTION

It is now generally agreed that the method of choice for the laboratory diagnosis of hepatitis A is to examine an acute phase serum for anti-hepatitis A immunoglobulin M (anti-HAV IgM) (Bradley *et al.* 1977). Specific IgM is usually demonstrable for several days before the onset of jaundice, and is almost invariably present by the time that jaundice appears (Frösner *et al.* 1979*b*). It can be shown to persist in diminishing amounts for up to six months (Mortimer, Vandervelde & Parry, 1979), and its presence in a serum collected during or after an icteric illness is diagnostic of recent HAV infection. Specific IgM is also found in association with subclinical and anicteric infections.

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Methods for detecting specific IgM fall into two groups, those in which IgM is isolated from the serum as a preliminary step, and those in which the specific IgM reactivity in a serum is measured selectively while other immunoglobulin activity is suppressed. Methods of the first kind involve the use of SDG centrifugation or staphylococcal protein A (Bradley *et al.* 1979) to separate IgM, which is then tested for anti-HAV. Methods of the second kind are based on an antiserum raised against the μ chain of human IgM. This reagent is coated onto the solid phase and reacts with IgM in the specimen in the initial step of the anti-HAV IgM test (Flehmig *et al.* 1979; Duermeyer, Wielaard & van der Veen, 1979).

During 1979 we carried out over 700 anti-HAV IgM tests, at first measuring anti-HAV in SDG separated IgM-rich serum fractions, then using this procedure and a method based on anti-μ serum bound to a solid phase in parallel, and finally using the latter test alone. We describe here our experience with these two tests for anti-HAV IgM.

MATERIALS

Sera to be tested for anti-HAV IgM were taken from clinical cases of hepatitis. They had already been examined for hepatitis B surface antigen by reverse passive haemagglutination (*Hepanosticon*, Organon) and RIA (*Ausria II*, Abbott) and, in some cases, for anti-hepatitis B core IgM (Mortimer *et al.* 1980), and found to be negative. Sera used in the evaluation of the anti-μ test included some from cases of infectious mononucleosis that had anti-Epstein Barr virus IgM (Edwards & McSwiggan, 1974) and some that were strongly positive in a latex test for rheumatoid factor (RF) (*Wellcotest*, Wellcome): these sera were supplied by Dr J. M. B. Edwards.

METHODS

All specimens were screened for anti-HAV by *HAVAB* (Abbott): sera with anti-HAV were then tested for anti-HAV IgM. Two SDG centrifugation methods were used to separate IgM. The first of these has already been described (Mortimer, Vandervelde & Parry, 1979). The second, re-orientation centrifugation, was introduced when a VTi.65 'vertical' rotor was loaned to us by Beckman, Ltd. Gradients of 40–13% w/v sucrose were prepared and 150 μl of each specimen were mixed with an equal volume of phosphate buffered saline (PBS) and layered onto the top of each gradient. The centrifuge tubes were sealed and spun in sets of eight in the vertical rotor. The speed was initially increased very slowly until 1000 rev./min was attained, and then more rapidly until 50000 rev./min was reached. After 90 minutes at that speed the drive was switched off and the rotor braked to 3000 rev./min. It was then allowed to coast to a standstill. An IgM-rich, IgG-free fraction from each gradient was selected by gel immunodiffusion testing of the fractions against anti-human IgM and IgG sera (Wellcome), and then tested for anti-HAV with and without treatment with 2 ME as described by Mortimer *et al.* (1979).

The anti-μ method used was similar to that of Flehmig *et al.* (1979). Polystyrene beads (6·4 mm diameter, Precision Ball Co., Chicago, U.S.A.) were coated by

immersing them for at least 18 h in a 1:500 dilution of sheep anti-human μ chain serum (Seward) in 0·01 M carbonate buffer, pH 9·6. Sufficient beads for each batch of tests were transferred to a conical flask, washed in PBS and immersed in anti-HAV negative human cord blood serum, 0·75% in PBS, for three hours at room temperature in order to block non-specific binding by IgG. Each specimen was diluted 1:10000 in PBS with 0·5% cord serum and 170 μl of it placed in a well of a plastic tray (Abbott). A bead was transferred from the flask to each well and the plate incubated for three hours at 37 °C. In each run anti-HAV IgM-positive and IgM-negative control sera, tested in triplicate, were included.

The beads were washed and 170 μl of HAV antigen, diluted in PBS containing 0·25% Tween 20 and 0·05% formaldehyde, was added to each well. The antigen for the anti-μ test was prepared from the faeces of marmosets, *Saguinus labiatus*, infected with the MSI strain of HAV. This strain was kindly supplied by Professor A. J. Zuckerman. Daily faecal collections were made up as 10% w/v suspensions in synthetic medium 199 (Wellcome), and tested for suitability by using them at various dilutions as the antigen in an anti-μ procedure in which control sera known to be positive and negative for anti-HAV IgM were substituted for the serum specimens. Faecal collections taken about day 10 of infection, and used in the form of a 1 in 10 to 1 in 25 dilution of the 10% suspension so that they gave a P:N ratio (count of positive control serum ÷ count of negative control serum) of $\geqslant 10$ were regarded as satisfactory.

The plates were incubated overnight at room temperature and for a further 24 h at 4 °C. The beads were then washed and labelled anti-HAV (approximately 300000 cpm in 170 μl) was added. Two labelled anti-HAV preparations were used. One was given by Dr R. S. Tedder, Department of Virology, Middlesex Hospital Medical School, and had been prepared according to the method of Bolton & Hunter (1973): the other was that supplied in the *HAVAB* kit. After three hours' incubation at 37 °C the beads were washed and their radioactivity measured. The results were expressed as T:N ratios, T being the specimen count and N the mean count of three tests on the anti-HAV IgM negative control serum. Sera with T-N values $\geqslant 5$ were reported as anti-HAV IgM positive and sera with T:N values < 2 negative. Sera with T:N values between 2 and 5 were regarded as equivocal.

RESULTS

The SDG method was used to test 384 sera. Separation of IgM from IgG, as shown by the results of immunodiffusion reactions with anti-IgM and IgG sera, was obtained in all sera, and was equally good by both centrifugation techniques. When an IgM-rich fraction was examined for anti-HAV IgM using the 2 ME reduction test 160 sera (41·7%) gave positive and 206 (53·6%) negative results (Table 1).

In 16 sera the anti-HAV reactivity of the IgM fractions was not completely removed by treatment with 2 ME. Ten of these sera were collected in the week of onset of jaundice (mean 2·9 days) and five of them more than six weeks after onset. In two sera (one collected early and one late in infection) the IgM fraction

Table 1. *Number and percentage of anti-HAV IgM tests positive and negative by SDG and anti-μ methods*

	Number sera examined	Positive	Negative	Equivocal
SDG	384	160 (41·7)	206 (53·6)	18 (4·7)
anti-μ	736	313 (42·5)	412 (56·0)	11 (1·5)

Table 2. *Thirty-two sera, not from cases of hepatitis A, tested to investigate possible non-specific reactivity in the anti-μ test (T:N < 2 ≡ negative, T:N value 2–5 ≡ equivocal)*

Category of serum	Number tested	Number anti-HAV positive	T:N ratios Range	T:N ratios Mean	Number with T:N ratio > 2
Paul Bunnell positive	4	1	0·78–1·26	0·97	—
Acute hepatitis B	4	2	0·90–1·29	1·09	—
HBsAg positive chronic hepatitis	5	4	0·88–1·00	0·95	—
HBsAg negative chronic hepatitis	3	3	0·90–2·27	1·44	1 (2·27)
Rheumatoid factor positive	14	11	0·90–2·11	1·21	1 (2·11)
High titre anti-HAV*	2	2	1·95–1·73	—	—

* Estimated titres by HAVAB 1/6000, 1/5000.

was anti-HAV negative, but the counts were close to the positive/negative cut-off for the *HAVAB* test and the activity was reduced by 2 ME treatment. The results on these 18 sera were regarded as equivocal.

With the anti-μ test initial investigations were performed to test the specificity of the method. Thirty-two sera were examined, 16 from cases of non-A hepatitis, 14 that were RF positive and two collected years after HAV infection but with unusually high titres of anti-HAV (Table 2). Two of these sera gave results marginally above the limit for a negative result (T:N values 2·27, 2·11). Most of the other sera gave T:N ratios close to unity. The ratios were not, on average, higher in those of the 32 sera that were anti-HAV positive.

The anti-μ method was used to test 736 sera, including 382 that had also been tested by the SDG method (Fig. 1). There were 313 (42·5%) positive, 412 (56·0%) negative and 11 (1·5%) equivocal results (Table 1). Equivocal results were of two kinds, five from sera that had been collected >80 days (mean 115 days) after illness with a T:N range of 2·54–4·20, and six from sera, collected at <80 days, that had T:N ratios only just above 2 (range 2·06–2·35).

There were 23 (6·0%) indefinite or discrepant results from the 382 tests performed by both methods (Table 3). All but three were of the form SDG equivocal or negative: anti-μ positive or equivocal. Most of these results occurred on sera collected <7 or >42 days after the onset of jaundice.

Fig. 1. Results of tests for anti-HAV IgM by the SDG and anti-μ methods on 738 sera found to be anti-HAV positive in preliminary tests.

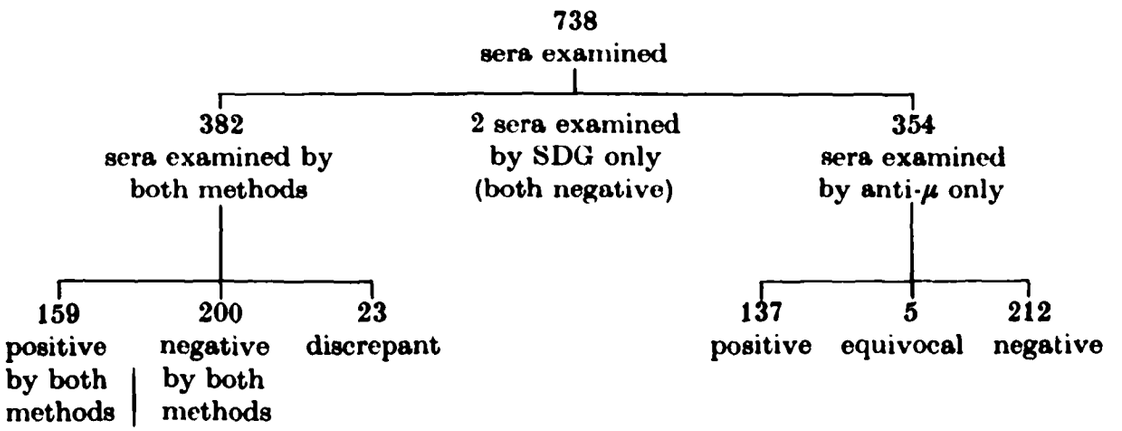

Table 3. *Discrepant and indefinite results arising from SDG and anti-μ tests for anti-HAV IgM on 382 sera*

Result		Number	
SDG	Anti-μ	sera	Remarks
Negative	Equivocal	3	Sera from day 20, day 80, day 127
Negative	Positive	1	Date of collection not known
Equivocal	Positive	16	10 sera from before day 7, 5 after day 42
Equivocal	Equivocal	2	
Positive	Equivocal	1	Insufficient serum to repeat SDG
Total		23	

DISCUSSION

Two methods are available to laboratories for the assay of anti-HAV IgM, and at present the choice between them depends more on local resources than on the intrinsic merits of the tests. While the SDG method described here can be used in any diagnostic laboratory that has access to an ultra-centrifuge and γ-counter, the anti-μ test can only be carried out if the laboratory possesses some HAV antigen. Supplies of this antigen are not abundant at the moment. This difficulty is soon likely to be overcome, however, either through the development of commercial 'anti-μ' tests, or by more HAV antigen from primate faeces or liver becoming directly available to laboratories. Tissue culture, too, may be a source of HAV antigen in the future (Frösner *et al.* 1979*a*).

The change from the SDG to the anti-μ method in our laboratory enabled us to make a comparison of the two in routine use. We were concerned with the relative cost and simplicity of the two methods as well as with their precision and sensitivity. In costing the two tests much depends on local circumstances. The SDG method is based on an expensive commercial anti-HAV test and entails the use of centrifugation equipment that is costly to buy and maintain. The anti-μ test is much cheaper overall than the SDG if a source of HAV in human faeces has been

found. On the other hand the cost of using a primate colony as a source of HAV is high. As regards convenience there is no doubt that the anti-μ test is better. The procedure is straightforward, and fewer manipulations are required than in the SDG, in which good results can only be obtained if the IgM separation is meticulously carried out.

The two density gradient procedures used were found equally effective in isolating serum IgM. The reorientation method allowed two runs of eight to be processed in a day, and would, for that reason, be preferable if the necessary equipment were available; but it offered no other advantage than speed and in view of its expense is unlikely to be widely used for some time.

Whichever density gradient procedure is employed problems may arise in the interpretation of anti-HAV tests on IgM-rich fractions. These stem from the fact that IgM does not inhibit the binding of labelled IgG very effectively in a competitive test of the *HAVAB* type. At best there is about 80% inhibition of label uptake by IgM fractions as opposed to >95% by whole sera or IgG fractions. It does not require much contaminating IgG to cause inhibition similar to that usually produced by IgM, and, for that reason, a 2 ME reduction procedure was included in tests on every IgM fraction examined in this series. As a result of using 2 ME, 16 sera were found in which a positive reaction could not be reversed by reduction of the IgM, and two in which a reaction close to, but beyond, the cut-off for the HAVAB test was modified by 2 ME. Sixteen of these 18 sera were anti-HAV IgM positive by the anti-μ test, and two equivocal, suggesting that in none of these cases was contaminating IgG rather than IgM being measured. However, all 18 specimens were collected unusually early or late in infection, and it seems that between day 4 and 42 of jaundice the 2 ME procedure will confirm an anti-HAV IgM positive reaction without causing any true positive results to be rejected because the results are not clear-cut. The 2 ME test is therefore a worthwhile check on the quality of SDG separation of sera and will not, in routine diagnostic use, give rise to falsely negative results.

Initial doubts about the specificity of the anti-μ test for anti-HAV IgM were not confirmed. It had seemed possible that false positive tests might arise in other forms of hepatitis, or when sera containing RF or high titres of non-IgM anti-HAV were examined. The series of non-A hepatitis, RF positive and high titred anti-HAV sera (Table 2) did not show this: nor did anti-HAV positive sera have higher T:N values than sera without anti-HAV. There was no indication from these results that anti-HAV not of the IgM class could interfere with the anti-μ test.

When the anti-μ test was introduced as a routine T:N values of 5 and 2 were arbitrarily set as the limits separating positive and equivocal from negative specimens. With a suitable HAV antigen T:N values for specimens collected in the acute phase of hepatitis A were between 10 and 20, and only sera collected more than two months after the onset of jaundice had T:N values <5. Although this method of reporting the results is satisfactory when a standardized preparation of antigen is used it would otherwise be too dependent on the potency of the antigen, and, for this reason, additional controls for anti-μ tests are now considered desirable. One simple means of controlling the tests is to select a weakly

anti-HAV IgM positive serum, divide and store it in small aliquots, and test it in triplicate with each batch of anti-μ tests so that it can be used as the serum giving the 'cut-off' value. Alternatively, the proportion of IgM in each specimen that is specifically anti-HAV can be compared to that in a strongly positive anti-HAV IgM control serum by testing dilutions of the control in an anti-HAV negative serum, plotting the dilutions against the counts bound, and relating the counts bound by each specimen to a dilution of the control serum. If a potency in units per ml is assigned to the control serum each result can then also be expressed in units per ml.

While the anti-μ results from the routine tests were in broad agreement with the SDG in this series (Fig. 1), more anti-μ results were positive on sera from the period before jaundice was established, and after recovery from the acute illness. A further indication that the anti-μ was the more sensitive test was the finding of equivocally positive anti-μ results before the onset of jaundice and more than three months after it. This extra sensitivity could account for most of the discrepancies between results by the two methods.

The real advantage of the anti-μ test was not, however, its sensitivity but the better discrimination it gave between positive and negative sera. This was lacking in the SDG-based test, in which sera apparently containing anti-HAV IgM sometimes failed to give clear-cut positive reactions in the HAVAB test. It is to be hoped that, either through the introduction of a commercial anti-μ kit, or through an increase in the supply of HAV antigen, the anti-μ test can be made freely available to diagnostic laboratories. There is no doubt that, because of its ease of performance, precision, sensitivity and potentially low cost, the anti-μ test is the method of choice for detecting anti-HAV IgM.

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