

The serology of delta hepatitis and the detection of IgM anti-HD by EIA using serum derived delta antigen

Alan G. Shauock¹, Marie Morris¹, Kevin Kinane²
and Ciaran Fagan²

¹Department of Medical Microbiology, University College, Dublin 4 and ²Noctech Ltd., Deans
Grange Industrial Estate. Co. Dublin, Ireland
(Accepted 28 August 1988)

Summary

A sensitive and specific capture assay for IgM antibody to hepatitis D virus (HDV) was developed employing serum-derived delta antigen (HDAg). In a retrospective and prospective study of an outbreak of hepatitis B (HB), 135 hepatitis B surface antigen (HBsAg) positive drug-abusers with acute hepatitis and 18 HBsAg carriers, attending various hospitals and clinics in Dublin, were found to be infected with HDV. Serological follow-up was available from 24 of those with acute hepatitis allowing a comparison of the duration and level of IgM anti-HD with the more commonly used markers. HDAg and anti-delta (anti-HD), and an assessment of the usefulness of each. HDV and HB serology was grossly altered by human immunodeficiency virus (HIV) in two patients, with severe clinical manifestation in one.

All 135 patients with HDV co-infection had delta antigenaemia. In co-infections with optimum sampling times, the mean duration of delta antigenaemia was 21 days. IgM anti-HD was always found between HDAg and sero-conversion to anti-delta and was the only 'window' marker present in five cases. The mean duration of IgM anti-HD was four weeks (optimum at 2.8 weeks) and was of moderate or low titre and occurred simultaneously with HDAg in 78%. In HDV-infected HBsAg carriers, high-titre IgM anti-HD (>1/10 000) persisted for the duration of the study and is a useful indicator of chronic HDV infection. IgM anti-HD was not found in 202 random blood donors nor in 205 patients with non-B hepatitis or other disorders. Total (IgG) anti-HD persisted in all patients (except the two reactiva-

Correspondence to: Alan G. Shattock. Dept. of Medical Microbiology, University College. Belfield. Dublin 4. Ireland.

0166-0934/89/£03.50 © 1989 Elsevier Science Publishers B.V. (Biomedical Division)

tions) at titres of less than 1/1000 in co-infections and greater than 1/5000 in carriers, for the duration of follow-up.

We conclude that HDAg is singly the best marker of HDV co-infection and, where only late specimens are available. IgM anti-HD is also useful. IgM anti-HD will also distinguish between chronic HDV infection and previous exposure.

Hepatitis D; Delta; HDAg; IgM-HD

Introduction

Co-infection or superinfection of hepatitis B with hepatitis D virus (HDV) may often result in an episode of acute hepatitis and, ideally, is best diagnosed by demonstrating virus or delta antigen (HDAg) in the serum. However, HDAg has been reported as transient and therefore easily missed if an early serum sample is not available (Rizzetto, 1983; Smedilie et al., 1983). This is not always the case if sensitive assays are used (Buti et al., 1986; Hoy et al., 1984; Shattock et al., 1985). Total anti-HD tests do not confirm acute infection unless the prior serological state of the patient is known. Furthermore, 'total' antibody tests are not optimised to detect IgM class antibodies. IgM specific anti-HD testing is a possible alternative means of diagnosing acute delta infection, before sero-conversion to IgG anti-HD (Aragona et al., 1987),

We now describe an IgM capture EIA for the detection of IgM anti-HD using HDAg extracted from serum as the antigen source and correlate the results of this assay with the other HDV markers.

Materials and Methods

Patients

HDV co-infection (anti-HBc IgM positive) was detected in 135 HBsAg positive patients during an outbreak of hepatitis B and HDV infection in drug-abusers which commenced in 1980 and continues to date (Shattock et al., 1982, Shattoek et al., 1985). HDV infection was also detected in 18 HBsAg carriers, three of which were HDV superinfections. These patients were all intravenous drug-abusers attending hospitals in Dublin and/or the Drug Advisory and Treatment Centre, Jervis Street, Dublin.

Detection of IgM anti-HD

Reagents

The solid-phase was prepared by coating microtitre strips or plates (Nunc, Immuno grade) with a 1/1000 dilution of rabbit anti-human IgM. u-chain specific an-

tibody (Dako) in sodium carbonate buffer, pH 9.5, overnight at 4°C and quenching with 0.5% bovine serum albumin (Sigma, crystalline Conn fraction V) in carbonate buffer. Solid phase plates were stored, either half filled with quenching solution at 4°C for up to a month, or dried, for long periods. High titre anti-HD (1/64000 by EIA, Noctech) was from a drug-abuser with chronic HDV infection and was purified and conjugated to horseradish peroxidase (Sigma type VI) as previously described (Shattock and Morgan, 1984) but without dinitrofluorobenzene. Serum HDAg reagent was obtained by pooling sera with an OD >1.0 for HDAg (tested by EIA as below). Only sterile sera, negative for rheumatoid factor (RA test, Medlabs, Dublin) were included in the reagent, which was aliquoted and stored at -20°C. The chromogen-substrate used was trimethyl benzidine (TMB) in citrate-phosphate buffer containing 0.01% hydrogen peroxide.

Test method

Each specimen was tested in a pair of wells, one with HDAg and one without HDAg as a control to detect non-specific activity. Sera, and positive and negative controls, were diluted 1/100 in Tris-saline, pH 7.4, containing 0.1% Tween-20 (TS) and added to test wells in 100 µl volumes, in pairs. Titrations were carried out in normal human serum (NHS), prior to 1/100 dilutions. Sera were incubated on the solid-phase for one hour at 37°C in a waterbath. Following washing four times with TS, 50 µl of HDAg reagent (diluted so as to give optimum sensitivity within the conditions of the test, by box-titration) was added to the first of each pair of wells, Fifty microlitre of NHS pool, negative for all HDV markers, was added to the second of each pair; 50 µl delta extraction buffer (Noctech) was then added to all test and control wells. Test plates were then incubated at room temperature overnight-After washing as before, 100 µl of anti-HD horseradish peroxidase conjugate was added to all wells followed by incubation for 1 h in a waterbath at 37°C. Substrate was incubated for 30 min at room temperature and the reaction was stopped by adding 100 µl of 4 N sulphuric acid to each well, Absorbance was read at 450 nm. The 'corrected optical density' (COD) for each specimen was calculated by subtracting the optical density (OD) of the control well from the corresponding antigen containing well. Samples repeatedly giving a COD of greater than 0.08 (calculated from three standard deviations above the mean CODs of 407 negative sera) were considered positive.

Specificity tests

The specificity of the conjugate for reaction with other hepatitis B antigens was determined as previously published (Shattock and Morgan, 1984). The reagents were also tested for reactivity with panels of sera from 13 patients with hepatitis A IgM antibody, 10 patients with antibody to hepatitis B core IgM, 5 patients with CMV IgM antibody, 11 patients with toxoplasma IgM antibody, 205 patients with other non-hepatitis B disorders, and a panel of 20 sera containing rheumatoid factor; 202 volunteer blood donors were also tested.

Other tests

Tests for serum HDAg and blocking (predominantly IgG) anti-HD were also by EIA (Noctech, Dublin) based on the method of Shattock and Morgan (1984) but using a modified HDAg extraction buffer which substantially raises the sensitivity. One unit of delta antigen was defined as the amount in 100 μ l of sample giving an OD⁴⁵⁰ of 0.5 in the test. All positives were confirmed by neutralisation. Tests for HBsAg (Abbott Labs) and IgM anti-HBc (in-house) were also carried out by EIA.

Results

IgM anti-HD capture assay - specificity and sensitivity

IgM anti-HD reactivity was only found in patients with previous or concomitant HDAg or with (total) anti-HD. It was not found in any of the control groups of sera with IgM antibodies to hepatitis A, hepatitis B, CMV or toxoplasma, or in 205 patients with other hepatitis B negative disorders- Blood donors (iV=202) were also unreactive as were the panel of sera containing rheumatoid factor. Some specimens with concomitant HDAg showed higher than average ODs in the control well, because of the indiginous HOAg. Such sera were positive in the HDAg capture assay.

HDV markers

The occurrence of HDV markers among co-infections is summarised in Table 1, The apparently different rates of sero-conversion to IgG or IgM or both are because of the difficulties experienced in obtaining follow-up specimens at regular intervals from drug-abusers. It was therefore necessary to divide the total HDV infected group into different, but overlapping groups according to specimen timing, in order to analyse the various HDV markers.

TABLE 1

Seroconversion patterns among 135 HDV co-infections by follow-up period

Follow-up period/s and	HDAg	Anti-HD		No. (%)
		IgM	IgG	
Acute only	+	-	-	50 ^a (37.0)
Acute and early convalescent	+	+	-	15 (11.1)
Acute and late convalescent only	+	-	+	25 (18.5)
Multiple specimens	+	+	+	37 (27.4)
Multiple specimens	+	-	+	8 (5.8)
Totals	135(100)	52(38)	70(52)	135

^aForty-eight of these did not return for any follow-up.

TABLE 2

Duration and level of HDV markers in patients with HDV/HBV co-infections and multiple specimen follow-up

Patients with:	No.	Mean duration	Range	Level/titre
HDAg. multiple specimens ^a	24	21 days	9-83 days	10.0-15000 units ^c
HDAg. All ^b	53	13 days	4-83 days	10.0-15000 units ^c
IgM anti-HD	23	4 weeks	2-20 weeks	<1/5000
Anti-HD (IgG)	34	25 months	3-73 months ^d	<1/1000

^a Patients with not more than 9 days between acute specimens.

^b All patients with more than one acute specimen.

^c One 'unit' of HDAg is defined as the amount of HDAg in 100 ul giving an OD of 0.5 in the assay.

^d No patient became negative during follow-up

Duration and. levels of HDV markers in delta co-infections

HDAg was the first marker detected in all 135 patients with delta co-infection, Of these, 53 had multiple acute specimens allowing a determination of the duration and peak levels of antigenaemia. Twenty-four of these had intervals not exceeding 9 days between specimens in the acute period, and the mean duration of antigenaemia in these was 21 days (range 9-83 days). The mean duration in all 53, irrespective of sampling dates was 13 days. Levels of HDAg ranged from. 10.0 to 15000 units/ml (Table 2). Sero-conversion patterns are shown in Table 1.

Of The same group of 24 with maximum 9 day intervals between acute specimens, 22 (92%) also had IgM anti-HD. The mean duration of IgM anti-HD was four weeks (range 2-20 weeks) with titres of < 1/5000. The mean (optimum) occurrence of IgM anti-HD was at 2.8 weeks after admission. The earliest occur-

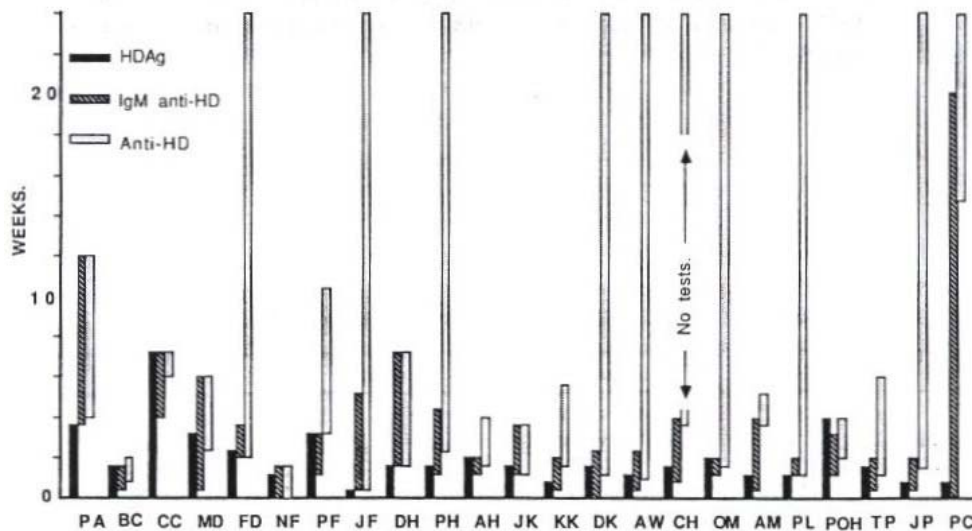


Fig. 1. Serological sequence in 23 patients with acute HDV co-infection.

rence was simultaneously with HDAg. The latest occurrence of IgM anti-HD in this group was 20 weeks after onset.

The serological sequence of HDV markers for 23 patients with HDV co-infections and IgM anti-HD is shown in Fig. 1, HDAg and IgM anti-delta were present simultaneously in 18 (78%) and IgM anti-HD was the only delta marker present (a 'window' marker) between the loss of HDAg and seroconversion to (IgG) anti-HD in 5 cases (23%).

IgM anti-HD was not detected in 8 (5,8%) of cases where specimens covering the late acute and early convalescent period were available and IgM anti-HD could reasonably be expected to occur.

Thirty-four patients had convalescent follow-up specimens in the range 3 months to six years and none of these lost anti-delta during this period (mean duration 2 years 1 month) although the titres were < 1/1000.

One patient, with an apparently 'normal' acute serological course and clearance of acute HBV markers and HDAg, reactivated both hepatitis B and hepatitis D a year later, again with delta antigenaemia. This patient was subsequently found to have been positive for antibody to human immunodeficiency virus (HIV) since his original HBV and HDV infection (Shattock et al., 1988).

Duration and level of HDV markers in HBsAg carriers

Of 18 HDV infected HBsAg carriers. 16 had follow-up serology (Table 3): one had been continuously positive for serum HDAg for over four years with fluctuating IgM anti-HD. He is also anti-HIV positive and is the index case of reactivation of HDV and HBV infection by HIV (Shattock et al., 1987). The remaining 15 had IgM anti-HD in high titre (>1/10000) for a mean follow-up period of 31 months (range 2-72 months). All remained positive throughout follow-up except one who lost IgM anti-HD immediately following liver transplant. All 16 had (IgG) anti-HD in high titre (1/5000-1/64000) for a mean follow-up period of 33 months (Table 3).

TABLE 3

Duration and level of HDV markers in 16 HDV infected HBsAg carriers

Patients with:	No.	Mean duration	Range	Level/titre
HDAg	1 ^a	44 months	-	2-10 units
IgM anti-HD	15	31 months ^b	2-72 months	10000-100000
Anti-HD (IgG)	16	33 months	4-72 months	5000-64000

^a Also infected with HIV and had fluctuating IgM anti-HD.

^b All patients remained positive for IgM anti-HD except one who became negative following liver transplant.

Discussion

The IgM anti-HD capture assay described, employing serum-derived HDAg, was sensitive and specific and obviates the need for liver as the antigen source. The control test, with no added antigen, was used to define the cut-off and to detect non-specific reactivity.

HDAg was the first and most frequent HDV marker detected in all 135 cases in this study. compared with a total of 52% with (IgG) anti-HD and 38.5% with IgM anti-HD overall, but biased by the non-return of patients for follow-up- This confirms that HDAg is the best marker for delta co-infection in drug-abusers, as Buti et al. (1986) have suggested. If HDAg testing had not been used 50 cases (37%) of HDV infection would not have been detected in our group. The mean duration of antigenaemia (21 days with multiple specimens, maximum 83 days) found by us is considerably more than the mean of two days (maximum 5 days) recorded by Aragona et al. (1987), and could be due to late hospital admissions or differences in test sensitivity due to the different extraction process used, or both.

We have noted recurrence of HDAg in one case and HDAg carriership for over four years in another, both infected with HIV. Both cases are regarded as reactivations of HDV by HIV (Shattock et al., 1987, 1988).

The majority (92%) of patients with co-infection with sufficient specimens showed sero-conversion to IgM anti-HD on average 2.8 weeks after admission, with a mean duration of four weeks, a finding similar to that of Aragona et al. (1987). However, in contrast to the same authors, we found no delays, or 'windows' between disappearance of HDAg and the appearance of IgM anti-HD. On the contrary, overlap between HDAg and IgM anti-HD occurred in 78% of cases. This was due to the presence of HDAg/IgM anti-HD complexes, detectable in the HDAg capture assay and also by capture in the IgM anti-HD assay control (data not shown). In 27% of cases IgM anti-HD was the only HDV marker present between HDAg and (IgG) anti-HD. This incidence, while not as great as that of Aragona et al. (1987) where almost all had an IgM-only 'window', is still sufficient to render IgM anti-HD a useful marker of acute HDV infection for patients where HDAg testing is not available or lacks sensitivity. The reason why IgM anti-HD was not detected in 5.8% of cases is unclear but is thought to be due to the fact that these sera were stored for long periods and had been repeatedly frozen and thawed.

The titres of IgM anti-HD were low ($<1/5000$) in acute co-infections compared with chronic HDV infections, where titres were very high (up to $1/100\ 000$) and lasted throughout the study. High titre IgM anti-HD therefore appears to be an excellent indicator of chronic HDV infection, as suggested by Farci et al. (1986), but our data do not support the view that IgM anti-HD is the best marker of self-limiting co-infection (Aragona et al., 1987).

Similarly, patients with chronic HDV infection had high (IgG) anti-HD titres compared with self-limiting co-infections, where lower but persistent (IgG) anti-HD was found. The duration of (IgG) anti-HD following our co-infections seems to be longer than previously reported by Aragona et al. (1987) and at complete variance with that reported by Smedilie et al. (1982) who found no IgG anti-HD

following acute self-limited HDV co-infection. We conclude that total (IgG) anti-HD is very useful as a marker of previous HDV exposure.

The serological sequences seen by us indicate that, for monitoring HDV co-infections, ideally, all markers of HDV infection should be tested for. Singly HDAg is the best marker of acute infection, unless only late specimens are available or expected, in which case IgM anti-HD would be useful in the majority of cases. IgM anti-HD will also distinguish patients with chronic HDV infection from those with previous exposure.

References

- Aragona, M., Caredda, F., Lavarini, C., Farci, P., Macagno, S., Crivelli, O., Maran, E., Purcell, R.H. and Rizzetto, M. (1987) Serological response to the hepatitis delta virus in hepatitis D. *Lancet* i. 478-480.
- Buti, M., Eateban, R., Jardi, R., Esteban, J.I. and Guardia, J. (1986) Serological diagnosis of acute delta hepatitis. *J. Med. Virol*, 18, 81-85.
- Farci, P., Gerin, J.L., Aragona, M., Lindsey, I., Crivelli, O., Balestrieri, A., Smedilie, A., Thomas, H. and Rizzetto, M. (1986) Diagnostic and prognostic significance of the IgM antibody to the hepatitis delta virus. *J. Am. Med. Assoc.* 255, 1443-1446.
- Hoy, J.F., Hansson, B.G., Dimitakakis, M., Gust, I., and Lucas, C.R. (1984) Delta agent infection in Melbourne. *J. Med. Virol.* 13, 339-345.
- Rizzetto, M. (1983) The delta agent, *Hepatology* 3, 729-737.
- Shattock, A.G., Kelly, M.G., Fielding, J.F. and Arthurs, Y. (1982) *Irish J. Med, Sci*, 151. 334-338.
- Shattock, A.G. and Morgan, B.M. (1984) Sensitive enzyme immunoassay for the detection of delta antigen and anti-delta, using serum as the delta antigen source. *J. Med, Virol*, 13, 73-82.
- Shattock, A.G., Irwin, F., Morgan, B.M., Hillary, I.B., Kelly, M.G., Fielding, J.F., Kelly, D.A. and Weir, D.G. (1985) Increased severity and morbidity of acute hepatitis in drug abusers with simultaneously acquired hepatitis B and hepatitis D virus infections, *Br, Med, J*, 290, 1377-1380.
- Shattock, A.G., Finlay, H. and Hillary, I.B. (1987) Possible reactivation of hepatitis D with chronic delta antigenaemia by human immunodeficiency virus, *Br. Med, J*, 294, 1656-1657.
- Shattock, A.G., Hillary, I.B., Mulcahy, F., Kelly, G. and O'Connor, J. (1988) Reactivation of hepatitis B and hepatitis D by human immunodeficiency virus. Poster and Abstract No. 4582, at IV International Conference on AIDS, Stockholm. June 1988.
- Smedilie, A., Lavarini, C., Crivelli, O., Raimondo, G., Fassone, M. and Rizzetto, M. (1982) Radioimmunoassay detection of IgM antibodies to the HBV-associated delta agent: clinical significance in delta infection. *J. Med. Virol*, 9, 131-138.
- Smedilie, A., Lavarini, C., Farci, P., Arico, S., Marinucci, G., Dentico, P., Giuliani, G., Cargnel, A., Blanco, C. and Rizzetto, M. (1983) Epidemiologic patterns of infection with the hepatitis B virus-associated delta agent in Italy. *Am. J. Epidemiol.* 117. 223-229.