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# IgM-antibody response to the hepatitis B core antigen in acute and chronic hepatitis B

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## SUMMARY

A solid phase M-antibody capture radioimmunoassay (MACRIA) and a serum fractionation method were used to quantitate the IgM response to the hepatitis B core antigen (IgM anti-HBc) in acute and chronic hepatitis B infections.

Antibody to the core antigen was predominantly of the IgM class during the acute phase of hepatitis B. Resolving acute infections remained positive by MACRIA, but at decreasing levels, for as long as 6 months. IgM anti-HBc persisted in HBsAg carriers but at levels very much lower than seen in acute infections. There was no correlation of IgM anti-HBc with severity of chronic liver disease in carriers.

Measurement of IgM anti-HBc by MACRIA enabled accurate identification of acute hepatitis B on single serum specimens.

## INTRODUCTION

Patients infected with hepatitis B virus (HBV) can be identified by the presence of hepatitis B surface antigen (HBsAg) in their serum. Most acute HBV infections resolve with clearance of HBsAg and development of antibody (anti-HBs) to it. However, a small number of patients remain persistently infected with HBV. HBsAg continues to circulate in their serum and these patients are termed carriers. In the United Kingdom HBsAg carriers are uncommon, comprising only 0·2% of the general population (Barbara *et al.* 1977). Thus a patient presenting with jaundice who is found to be HBsAg positive is most likely to have acute hepatitis B. However, in areas of the world where carriers are more common, it is not possible by testing a single specimen of serum from the ill patient to be sure that the jaundice in an HBsAg-positive person is due to hepatitis B. Jaundice may arise in carriers for many reasons unrelated to infection with HBV, e.g. drugs and gallstones. Similarly, finding HBsAg in the serum of a person without symptoms could indicate either an acute inapparent infection or the carrier state. Distinguishing the two is important since it may alter the practical management of the individual (Tedder, 1980). At present the diagnosis of acute HBV infection relies upon the demonstration of changing levels of HBsAg. It may take weeks for significant changes to occur, and this might delay the recognition of another cause of jaundice in an otherwise asymptomatic HBsAg carrier.

During the course of acute HBV infection antibody to the core antigen (HBcAg)

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develops. This antibody (anti-HBc) is a reliable marker for past or present virus replication. It has been shown that IgM anti-HBc can be demonstrated in the serum of patients with acute hepatitis B (Cohen, 1978) by serum fractionation followed by assay of the IgM- and IgG-rich fractions for anti-HBc. Recently a solid-phase fractionation radioimmunoassay for IgM antibodies to hepatitis A virus (IgM anti-HAV) has been described (Flehmig *et al.* 1979). An enzyme immunoassay based on a similar principle has been used to detect IgM anti-HBc (Gerlich & Luer, 1979) and an analogous M-antibody capture radioimmunoassay (MACRIA) has also been developed (Tedder & Wilson-Croome, 1980).

We report here the use of MACRIA for detecting IgM anti-HBc in sera from patients with acute hepatitis B, in chronic asymptomatic HBsAg carriers and in HBsAg-positive patients with chronic liver disease. In addition we have determined the time course of reactivity for IgM anti-HBc in acute hepatitis B infection. Preliminary results on reactivity of the MACRIA in acute hepatitis B infection have already been reported (Mortimer *et al.* 1980). In order to confirm the specificity of this assay for IgM anti-HBc detection, some sera in this study were also assayed for IgM antibody by the method using gel-filtration for serum fractionation.

## MATERIALS AND METHODS

### *Sera*

Serum samples were taken from patients with acute and chronic hepatitis B and stored at −20 °C. Aliquots of sera from HBsAg carriers with histologically confirmed liver disease were made available by Dr A. L. W. F. Eddleston, King's College Hospital, London.

### *Serum fractionation*

Serum was separated into IgM and IgG fractions by filtration on a column (40 cm × 2·6 cm diameter) packed with Sephacryl S300 (Pharmacia Ltd) as previously described (Tedder & Wilson-Croome, in press).

### *Competitive radioimmunoassay*

Portions of serum, serum fractions or dilutions thereof (50 $\mu$l volume), were mixed with an equal volume of $^{125}$I-labelled anti-HBc IgG (20 nCi activity) and incubated in HBcAg-coated tubes. After overnight incubation the tubes were washed and bound radioactivity estimated in a 16-channel $\gamma$-counter (NE 1600, Nuclear Enterprises Ltd, Edinburgh). Anti-HBc in the test material significantly reduced the binding of radioactivity (Tedder & Wilson-Croome, 1981). Serum or serum fractions were considered to contain anti-HBc if there was greater than 50 % inhibition of binding of $^{125}$I anti-HBc. Fractions of sera were diluted twofold in this assay. The reciprocal dilution giving 50 % inhibition, expressed as 50 % inhibitory RIA units, determined the amount of anti-HBc contained in that fraction. Presence of IgM antibody was confirmed by its sensitivity to reduction by 10 mM dithiothreitol for 1 h followed by alkylation with a 25 % equivalent excess of iodoacetamide (Tedder & Wilson-Croome, 1981).

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*M-antibody capture radioimmunoassay (MACRIA)*

Sera, or dilutions of sera in normal human serum (NHS, negative for all HBV markers) were diluted 1 in 2000 in phosphate-buffered saline containing 0·5 % BSA and 0·05 % Tween (PBSA-Tween) and assayed for IgM anti-HBc as previously described (Tedder & Wilson-Croome, 1981). Briefly, 100 $\mu$l volumes were incubated in tubes coated with IgG from sheep serum raised against the $\mu$-chain of human IgM. After incubation the tubes were washed and 100 $\mu$l volumes of CsCl-gradient purified HBcAg, diluted in PBSA-Tween, added to the tubes for a further incubation. The tubes were again washed and 30 nCi $^{125}$I anti-HBc were added in PBSA-Tween containing 5 % sheep and 1 % human serum. After a final incubation the tubes were washed and the binding of radioactivity measured. The proportion of the IgM immunoglobulin captured in the first stage which is specific anti-HBc determines the capacity of the solid-phase in the second stage for HBcAg and, ultimately, the uptake of $^{125}$I anti-HBc in the final stage. If the dilution of the assay is kept constant (1 in 2000 in PBSA-Tween) the level of $^{125}$I anti-HBc binding is directly proportional to the relative amount of IgM anti-HBc present in the test serum. Thus, it is possible to measure the reactivity of a specimen in MACRIA and assign it a level of IgM anti-HBc by comparison against a calibration curve of standard sera containing known quantities of IgM anti-HBc (Tedder & Wilson-Croome, 1981). A serum highly reactive in MACRIA was assigned an activity of 1000 arbitrary IgM anti-HBc units per ml. Dilutions of this serum in NHS were prepared to contain a nominal 100, 33, 10 and 3·3 units per ml IgM anti-HBc. These standards were run in each assay in order to provide a calibration curve for the quantification of test sera reactive in MACRIA. Standards containing 100 units per ml usually bound 20- to 40-fold more $^{125}$I anti-HBc than did NHS (P/N ratio 20–40 × NHS). The P/N ratio given by 3·3 units per ml (usually 3–6 × NHS) was considered to be the lower limit for detection of specific IgM anti-HBc and determined the cut-off for each assay. Test sera containing > 100 units per ml IgM anti-HBc were re-assayed after a preliminary dilution in NHS.

Sera from carriers frequently have very high levels of anti-HBc, sometimes amounting to 15–20 % of the total IgG. In these circumstances the non-specific adsorption of only small quantities of IgG to the solid phase could cause false MACRIA reactivity. It has been shown (Tedder & Wilson-Croome, 1981) that reactivity due to IgM anti-HBc can be confirmed by its sensitivity to inhibition by the addition of an excess of anti-$\mu$ sheep IgG to the first stage of the incubation. This procedure is termed the '$\mu$-block', and the resulting reduction in counts, '$\mu$-specific' counts, is related to the quantity of IgM anti-HBc in the test serum. Residual reactivity un-affected by the '$\mu$-block' (which was always less than 3·3 units per ml) is related to the total anti-HBc titre of the serum.

*HBsAg*

This was assayed by either reverse passive haemagglutination (Hepatest, Wellcome Reagents Ltd) or a sensitive solid-phase radioimmunoassay (Cameron *et al.* 1980).

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*HBeAg and anti-HBe*

These were detected by a solid-phase radioimmunoassay as previously described (Barbara *et al.* 1979).

*Anti-HBs*

This antibody was detected by a solid-phase radioimmunoassay. Polystyrene tubes (GW3, Seward Laboratory) were coated with purified HBsAg and 100 $\mu$l of test specimens or controls incubated in the tubes overnight at room temperature. After washing, the tubes were incubated with $^{125}$I-labelled HBsAg, finally washed and then counted for radioactivity. Sera containing $\geqslant$ 0·01 units per ml anti-HBs were considered positive (WHO standard).

## RESULTS

IgM anti-HBc was detected in acutely infected patients with both icteric and inapparent illness. A total of 109 sera from 43 patients taken during the acute and convalescent phases of resolving HBV infection were assayed by MACRIA and the IgM anti-HBc expressed in arbitrary units per ml. Sera were taken at the presentation of the patient (day 1). Approximately half these patients were either jaundiced on examination or had symptoms suggestive of acute hepatitis. Further specimens were collected during the course of the infection (days from onset). IgM anti-HBc was detected early in the illness and all but one patient had presenting levels greater than 50 units per ml (Fig. 1). The majority of sera contained IgM anti-HBc at levels between 100 and 500 units per ml, although in a few very high levels were found. IgM anti-HBc remained detectable by MACRIA for at least three months. In some instances the MACRIA was still positive six months after the onset of illness, by which time the patient's serum was either HBsAg-negative or contained anti-HBs.

Fractionation of sera and testing by competitive RIA for anti-HBc also demonstrated that IgM antibody was present early and that it declined slowly as the IgG antibody response developed. Five patients were sampled during the course of their illness and in the convalescent phase. Early in the course of the illness, anti-HBc was predominantly of the IgM class. This was emphasized if the results of serum fractionation were expressed as a percentage of the total anti-HBc (Table). In all early sera examined from the five patients (and in those from five other patients with acute, clinically apparent infections) IgM anti-HBc comprised the bulk of total anti-HBc antibody; it could be detected by both methods for up to three months but at this stage comprised only a small part of the total anti-HBc response.

Levels of IgM anti-HBc were measured by both methods in 34 sera from 14 patients and the results correlated well (Fig. 2). There was an indication that the MACRIA might be the more sensitive test. This was confirmed when the standard IgM anti-HBc sera were assayed by both methods. The lower limit of detection by the method involving serum fractionation was 10 MACRIA units per ml.

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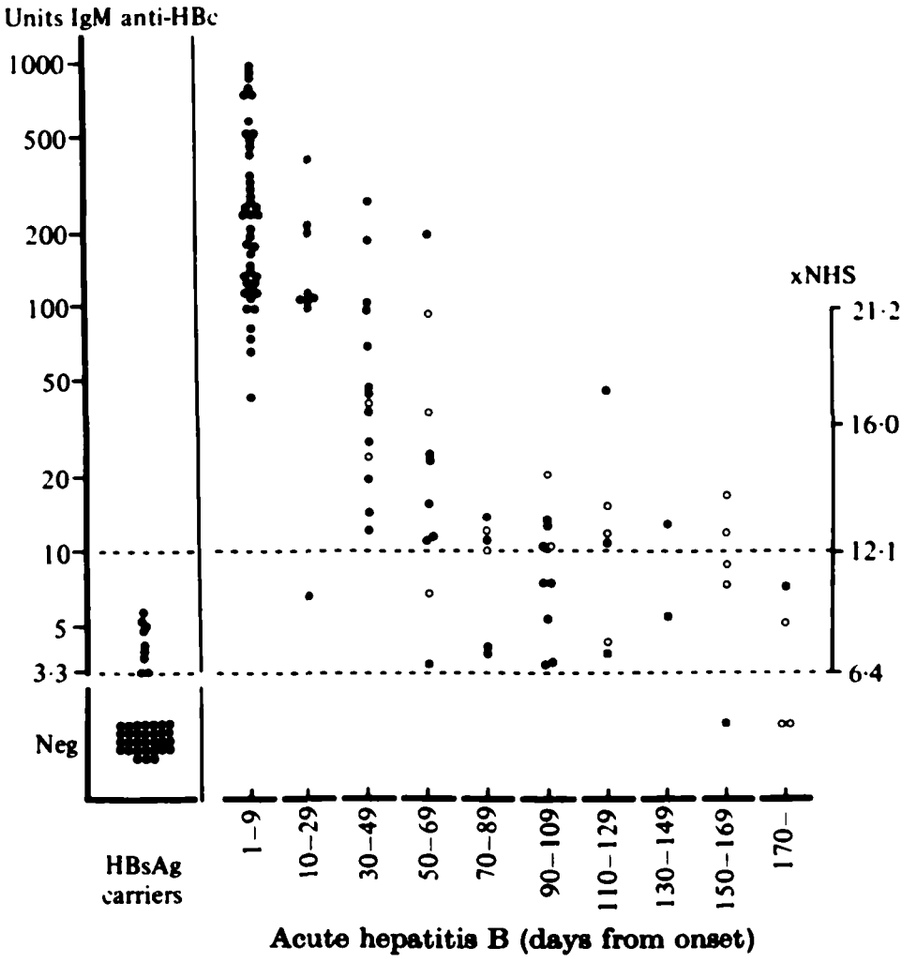


Fig. 1. IgM anti-HBc measured by MACRIA in 109 sera from 43 patients with acute hepatitis B during the early and convalescent periods. Results with sera from 40 asymptomatic carriers are included for comparison. ●, HBsAg +ve serum; ○, HBsAg/anti-HBs −ve serum; ■, anti-HBs +ve serum.

Table. *Anti-HBc response in five patients with hepatitis B*

| | | | Competitive RIA* | | |
|---|---|---|---|---|---|
| Patient | Day of illness | MACRIA units | IgM units | IgG units | IgM as percentage of total anti-HBc |
| WH | 1 | 760 | 57 | 5 | 92 |
| | 33 | 280 | 42 | 15 | 72 |
| | 100 | 13·5 | 2 | 44 | 4 |
| BT | 1 | 180 | 32 | 8 | 80 |
| | 28 | 200 | 44 | 16 | 73 |
| | 61 | 11·5 | 3 | 20 | 13 |
| | 95 | 7·5 | NT | NT | |
| RJ | 1 | 100 | 26 | 7 | 79 |
| | 48 | 27 | 8 | 66 | 11 |
| | 109 | 9·5 | 6 | 70 | 8 |
| BC | 1 | 760 | 256 | 20 | 93 |
| | 20 | 220 | 88 | 72 | 55 |
| | 44 | 104 | 10 | 36 | 22 |
| | 106 | 10·5 | 0 | 20 | < 1 |
| HV | 1 | 780 | 192 | 32 | 86 |
| | 32 | 96 | 32 | 144 | 18 |
| | 102 | 5·2 | 4 | 72 | 5 |

* Activity expressed as RIA 50% inhibitory units.
Coefficient of correlation, MACRIA units and competitive RIA IgM units = 0·71.
NT = not tested.

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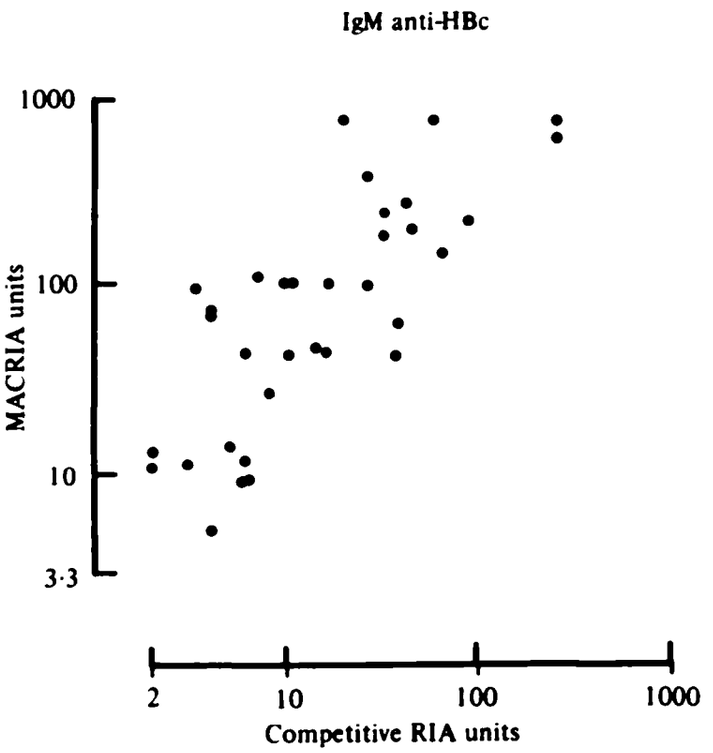


Fig. 2. IgM anti-HBc measured by MACRIA and serum fractionation in 34 single sera from 14 patients with acute hepatitis B.

Two additional patients suffered acute HBV infection which persisted and they developed into carriers. IgM anti-HBc in these individuals showed an early decline, but in both reactivity persisted at low levels during the early part of their carrier state for periods of more than a year.

Sera from ten patients with icteric hepatitis A (IgM anti-HAV-positive) were assayed for IgM anti-HBc. One serum had reactivity in the MACRIA equivalent to 3·6 units per ml, the remaining nine were negative (< 3·3 units per ml). Sera from 100 HBsAg-negative blood donors were similarly tested, and a single serum with no other HBV markers had slight reactivity, equivalent to 4·4 units per ml. Sera from 16 patients with non-HBV chronic active hepatitis (CAH; negative for HBsAg, anti-HBs and anti-HBc by RIA) did not have detectable levels of IgM anti-HBc although the mean count binding was higher than that of the 100 normal donor sera (P/N = 1·62, range 0·65–5·7 compared with P/N = 1·09, range 0·57–6·01).

To determine the level of IgM anti-HBc in chronic HBV infection, sera from 20 HBeAg-positive carriers, 20 anti-HBe carriers and 48 HBsAg-positive cases of chronic liver disease were assayed by both methods. Although histological confirmation of liver pathology was available for patients with chronic liver disease, the 40 carriers examined were asymptomatic and had not been biopsied.

None of the asymptomatic carriers had detectable IgM anti-HBc (IgM activity < 0·2 % of total anti-HBc) by the less sensitive serum fractionation method. Low levels of IgM anti-HBc were detected by MACRIA in HBeAg-positive carriers, but only nine were above the arbitrary cut-off of 3·3 units per ml (m = 4·4 units per ml, range 3·3–5·7). However, sub-threshold levels of IgM anti-HBc present in

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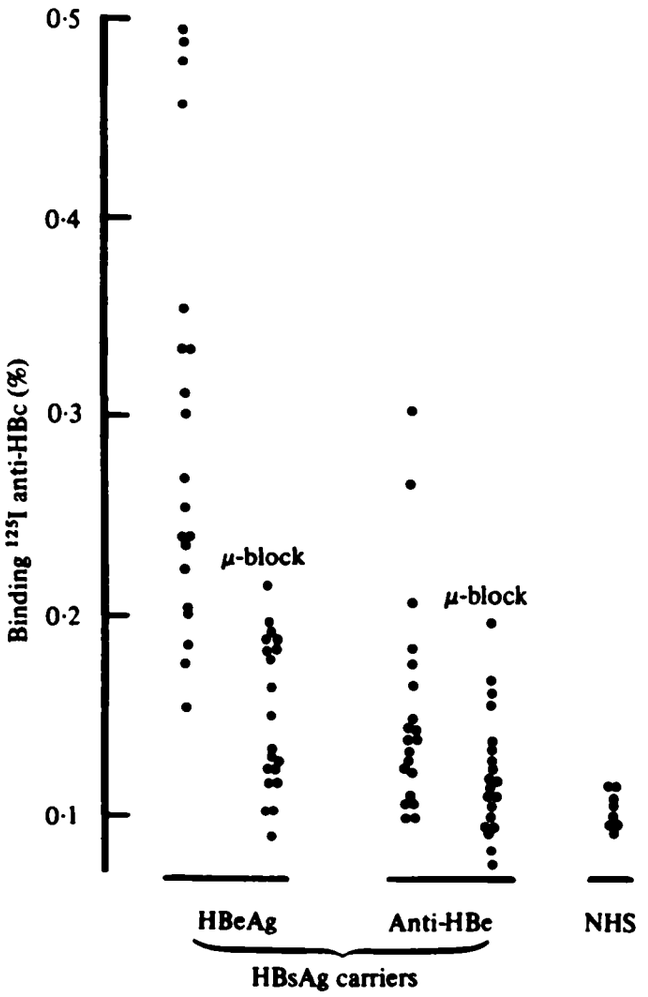


**Fig. 3. Effect of excess anti-$\mu$ serum ('$\mu$-block') in the first step of MACRIA on reactivity of sera from 20 HBeAg-positive and 20 anti-HBe-positive HBsAg carriers.**

the remaining HBeAg and many of the anti-HBe carriers could be detected by comparing the reactivity of sera in MACRIA with and without the '$\mu$-block' (Fig. 3). The reduction of reactivity brought about by the '$\mu$-block' was less marked in anti-HBe-positive carriers than in HBe-positive carriers.

Sera from 48 HBsAg-positive patients with histologically confirmed chronic liver disease were tested by MACRIA (insufficient serum was available for determining their HBe status). Of 8 patients with chronic persistent hepatitis (CPH) one had detectable IgM anti-HBc (5 units per ml). Ten of 34 patients with chronic active hepatitis (CAH) had detectable IgM anti-HBc (m = 4·8 units per ml, range 3·3–9·5) and this antibody was also detectable (3·3 units per ml) in one of six patients with cirrhosis. Although there was a higher prevalence of reactivity in patients with CAH it is not possible to comment on the significance of this finding without knowing their HBe status.

## DISCUSSION

Two different assays have shown that, in patients with acute hepatitis B, IgM anti-HBc was present in sera taken at or around the onset of jaundice. The method of gel filtration enabled the relative proportion of IgM or IgG anti-HBc

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to be determined. In this way it was shown that anti-HBc was predominantly IgM antibody early in the illness. Similar results were found by Cohen (1978) who used the relatively insensitive method of counter-electrophoresis to detect anti-HBc in IgM- and IgG-rich fractions. In contrast Brzosko *et al.* (1975) remarked on the apparent failure of hepatitis B core to elicit a prominent IgM response. The latter authors were using indirect immunofluorescence to detect anti-HBc. It is possible that blocking by IgG antibody might have masked the IgM response.

Class-specific antibody-capture assays are difficult to quantitate precisely as they indicate that proportion of the captured immunoglobulin which is specific antibody. Thus the activity of a serum in such a test may be influenced by fluctuation in the level of total serum IgM without changes in the level of specific IgM. Alternatively it may be possible for one serum to contain four times as much specific IgM antibody as another serum, but yet to have the same reactivity in MACRIA because the total IgM is also elevated fourfold. In spite of these theoretical objections to the method used to quantitate the MACRIA, the results by the two methods showed good agreement.

It was possible to distinguish between acute and chronic hepatitis B infections on the basis of IgM anti-HBc levels. Predominance of IgM over IgG anti-HBc was the most reliable marker of acute hepatitis B. The quantitative relationship of the classes of anti-HBc should be similar irrespective of the magnitude of the total anti-HBc response. This information was only given by the serum fractionation method, as MACRIA determined the IgM anti-HBc levels alone. Nevertheless, in most cases MACRIA will discriminate effectively, as in all cases studied except one the sera taken from patients when they first presented with acute infection had IgM anti-HBc levels above 100 units per ml. This range was clearly higher than the levels of IgM antibody present in HBsAg carriers. Similar results have recently been described using an analogous enzyme-linked immunoassay for IgM anti-HBc (Gerlich & Luer, 1979). However, earlier reports, using indirect immunofluorescence, indicated that IgM anti-HBc persisted for long periods in carriers at levels similar to those found in acute cases (Niermeijer *et al.* 1978). Rheumatoid Factor (RF) is common in sera from HBsAg carriers (Tedder & Briggs, 1977) and no attempt was made by Niermeijer and his colleagues to exclude the possibility of RF interfering with the immunofluorescence tests for IgM antibody.

The low levels of MACRIA reactivity present in sera from HBsAg carriers may be due to minimal quantities of IgM anti-HBc. However, the finding of occasional sera negative for anti-HBc and other markers of hepatitis B infection which showed some reactivity in MACRIA would indicate that there may be reactive components in the HBcAg preparation other than core. The occurrence of such HBcAg-reactive sera impose a limitation on how far the sensitivity of MACRIA can be improved. The majority of these reactions can be disregarded in practice by taking a 'cut-off' level of IgM anti-HBc which gives a ratio between 3 and 6 times NHS; this corresponded to a 1 in 300 dilution in NHS of the IgM-positive serum, nominally 3·3 units per ml IgM, and was used in each MACRIA assay to determine and standardize the lower-limit cut-off.

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IgM anti-HBc reactivity below the cut-off was regarded as negative, and low levels of IgM anti-HBc in the range 10–3·3 units per ml were viewed with caution, because although RF alone does not cause reactivity in MACRIA, it has been shown previously that activity equivalent to low levels of IgM anti-HBc could be generated by the combined presence of aggregated anti-HBc and RF (Tedder & Wilson-Croome, 1980). The reactivity found with anti-HBc-negative sera from two patients who did not have hepatitis B (one with hepatitis A and one asymptomatic blood donor) was modest and in both cases below 10 units per ml.

Low but significant levels of IgM anti-HBc were most commonly found in HBeAg-positive carriers, a group in whom mild CAH might be found more frequently. Although IgM anti-HBc was more prevalent in the CAH group, there was little to support a correlation between serious liver disease and an increased level of IgM anti-HBc. It appeared that IgM anti-HBc could not be used to indicate accurately the nature and severity of liver disease in HBsAg carriers.

The diagnosis of an acute infection by detection of IgM antibody depends upon an adequate host response. Sensitive assays may detect HBsAg up to months before the patient develops jaundice, at a time when anti-HBc has not yet been produced. The failure to detect IgM anti-HBc in sera taken at this time could erroneously be considered to indicate the carrier state unless the total absence of anti-HBc was recognized. In most acute cases of hepatitis B anti-HBc will be present by the time jaundice occurs. A negative MACRIA in an icteric patient, therefore, effectively excludes acute hepatitis B. Conversely, the failure to detect IgM anti-HBc in a serum found by chance to be HBsAg-positive does not exclude inapparent acute hepatitis B infection unless that serum is also positive for anti-HBc. Any serum which is to be tested for IgM anti-HBc should also be assayed for 'total' anti-HBc. Similarly, anything which interferes with this response, such as immunosuppression, may inhibit the development of reactivity in MACRIA. When an infection is attenuated, for example by the passive administration of antibody, virus replication and subsequent antigen challenge may be diminished, resulting in a reduced antibody response. Thus in patients given hepatitis B immunoglobulin (HBIG), infection might not result in the normal IgM anti-HBc response. In addition, the passive acquisition of anti-HBc, either in HBIG, transplacentally from a carrier mother, or in an infecting blood donation might inhibit the active anti-HBc response. It remains to be seen whether patients in these groups show an attenuated IgM anti-HBc MACRIA reactivity.

In many patients IgM anti-HBc could be detected by MACRIA at a time when HBsAg had disappeared from the serum, anti-HBs had not yet developed and anti-HBc was the only marker of previous infection. This finding may be of value in two situations. First, the use of MACRIA to detect IgM anti-HBc may be a useful adjunct in the investigation of blood donors involved in post-transfusion hepatitis B. Often by the time the implicated donors are recalled any acute case would have become HBsAg-negative, and the demonstration of IgM anti-HBc might be the only indicator of recent recovery from hepatitis B. Secondly, where the clearance of HBsAg is very rapid, as in fulminant hepatitis B, diagnostically high levels of IgM anti-HBc will be present in the serum. Thus, in these situations



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and in routine serology, the MACRIA for IgM anti-HBc can be used to diagnose acute hepatitis B on a single serum specimen. Even in the U.K. where the HBsAg carrier rate is low, the use of this test will avoid the delay brought about by the current requirement of paired sera for the accurate diagnosis of acute hepatitis B.

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