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Production of monoclonal antibodies to hepatitis B surface and core antigens, and use in the detection of viral antigens in liver biopsies

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SUMMARY

Hybridomas secreting monoclonal antibodies to HBsAg and HBcAg were prepared from immunized mice. An antibody capture radioimmunoassay was used to detect and select appropriate hybrids for propagation and cloning. The advantages of this assay were discussed. The resulting monoclonal antibodies were compared with conventional polyclonal antisera for the detection of virus antigens in liver tissue and found to give excellent results.

INTRODUCTION

Diagnosis of infection with the hepatitis B virus (HBV) relies predominantly upon the detection of the surface antigen (HBsAg) in serum. This antigen and the hepatitis B core antigen (HBcAg) can also be demonstrated within hepatocytes (Shikata, 1973; Yamada & Nakane, 1977). It has been suggested that certain antigen patterns may be associated with particular categories of liver pathology (Gudat *et al.* 1975; Ray *et al.* 1976; Nowoslawski *et al.* 1975) and that immunohistological examination of liver biopsies can be a useful adjunct to conventional histology. Early studies on HBV expression were conducted on frozen sections using immunofluorescent techniques, but it is possible to use rehydrated paraffin-embedded sections (Ray & Desmet, 1975; Huang, 1975; Portmann *et al.* 1976).

Conventional polyclonal sera from hyperimmunized animals or selected sera from humans have been used in the past for these investigations. Sera from both of these sources will inevitably contain a high proportion of redundant IgG which sometimes may give rise to unwanted and non-specific staining. When human sera have to be used, as is usually the case in the detection of HBcAg, an additional problem of tissue fixation of homologous antibody may arise.

The production of pure specific antibody to HBsAg (anti-HBs) and HBcAg (anti-HBc) from immortalized immunocompetent splenic lymphocytes (Köhler & Milstein, 1977) could provide a source of antibody which would overcome some of the problems associated with conventional sera. A limited study was undertaken using monoclonal anti-HBs and anti-HBc to detect HBV antigens in human liver

biopsies. Clones of antibody-secreting cells producing IgG antibodies of these specificities were derived from immunized mice and propagated as ascitic tumours. The resulting ascitic fluids containing high titres of specific antibody were used in indirect immunofluorescent and immunoperoxidase techniques to demonstrate HBsAg and HBcAg. The expression of these antigens was also investigated using conventional animal and human antisera.

METHODS

Animals

BalbC mice bred in the Bland Sutton Institute of Pathology, Middlesex Hospital Medical School or by Bantin and Kingman Ltd were used throughout this study.

Immunization

Twelve- to 14-week-old animals were initially immunized intra-peritoneally (i.p.) with purified antigens in 500 μl PBS mixed with an equal volume of complete Freund's adjuvant. Two further doses of antigen in PBS were given i.p. and a final dose given intravenously 3 days before fusion.

Fusion and propagation

The methods used differed little from other published techniques and have already been described in detail (Tedder, Yao & Anderson, 1982). Simply, spleen lymphocytes from immunized mice were fused with JKAg8 myeloma cells using 50% polyethylene glycol (BDH Ltd) and the resulting hybrids grown on feeder cells in selective medium. Antibody-producing hybrids were cloned by limiting dilution. The resulting monoclonal hybridomas were propagated as ascitic tumours in female BalbC mice which had previously been primed with an i.p. dose of 0·5 ml Pristane.

Testing of supernatant and ascitic fluids for specific antibody

Anti-HBs. Viable hybrids were screened for the production of anti-HBs by radioimmunoassay (RIA) employing a solid-phase of remova-well strips (Dynatech Ltd) coated with purified HBsAg (Tedder *et al.* 1980). One hundred microlitres of supernatant fluid (SNF) or dilution of SNF in 0·02 M TRIS, pH 7·6 containing 0·1% sodium azide and 0·5% bovine serum albumen (TBSA), were incubated overnight at room temperature (r.t.) in coated wells. These were then washed and incubated for 4 h at r.t. with iodinated ^{125}I-HBsAg. Antibody-positive hybrids and clones were tested in a solid-phase antibody capture radio-immunoassay (ACRIA) similar in principle to an enzyme immunoassay described by Boniolo, Dovis and Matteja (1982). Wells were coated with rabbit antibody to whole mouse IgG or class specific Fc. Dilutions of SNF 1:10 in TBSA were incubated overnight in coated wells at r.t.; the wells were then washed and incubated with 100 μl of differing HBsAg subtypes at a concentration of 100 ng HBsAg per ml (quantified in RIA using polyvalent antisera) in TBSA for 2 h at 37 °C. The wells were then washed and incubated with 100 μl affinity-purified rabbit ^{125}I anti-HBs IgG in TBSA containing 5% normal rabbit serum (NRS) for a further 2 h at 37 °C. The wells were then washed and binding of label measured in a sixteen-well γ counter (NE 1600, Nuclear Enterprises Ltd).

Anti-HBc. Viable hybrids were screened for the production of anti-HBc by testing SNF diluted 1:10 in TBSA in ACRIA as described above but using in this case HBcAg diluted in TBSA as the antigen and human ^{125}I anti-HBc IgG, diluted in TBSA containing 2·5% normal human serum (NHS) and 2·5% NRS, as the radiolabelled antibody. The SNF and ascitic fluids derived from anti-HBc-producing clones selected by ACRIA were later tested for reactivity in a solid-phase simultaneous competitive RIA for anti-HBc as described previously (Tedder *et al.* 1980).

Liver Biopsy

For this investigation liver biopsies from six patients were selected in which either HBsAg or HBcAg had previously been localized in liver cells both by immunofluorescence and immunoperoxidase techniques. The histological diagnosis was active cirrhosis (one case), chronic active hepatitis (one case), chronic persistent hepatitis (one case) and the hepatitis carrier state with minimal hepatic changes (three cases). Six other liver biopsies were chosen from HBsAg-negative patients with a miscellany of liver diseases unrelated to HBV (no detectable viral antigens in serum and liver).

All liver tissue was fixed in 10% formal saline for 12 h then dehydrated and embedded in paraffin. Sections 6 μ thick were heated for a few minutes at 56 °C, then deparaffinized through xylol and alcohol. Frozen sections were also available from one HBsAg-positive biopsy.

Immunohistochemical procedures

Tissue sections were tested with indirect immunofluorescence and indirect immunoperoxidase techniques for the presence of HBsAg and HBcAg.

Indirect immunofluorescence. A conventional technique was used. Sections were incubated with the optimal dilution of polyclonal or monoclonal antibody in phosphate-buffered saline (pH 7·4, PBS) for 30 min in a humid chamber at r.t. After washing with PBS, sections were reacted with FITC donkey anti-mouse IgG (Ortho Laboratories Ltd) for 30 min. Sections were washed and then finally mounted in PBS glycerin.

Immunoperoxidase. Endogenous peroxidase was blocked by treatment for 15 min with acid alcohol. Sections were washed in Tris saline buffer, pH 7·3, and incubated with the optimal dilution of mouse monoclonal anti-HBs or anti-HBc, or human anti-HBc for 45 min in a humid chamber at r.t. After washing, sections were reacted with a 1:30 dilution of horseradish peroxidase (HRPO)-conjugated rabbit anti-mouse IgG (Miles Laboratories Ltd) or HRPO-conjugated rabbit anti-human IgG (Dako Laboratories Ltd) as appropriate for 30 min. After reaction with diamino benzidine (DAB) sections were extensively washed in running tap water, counter-stained with diluted Mayer's Haemalum, dehydrated, cleared and mounted in Canada Balsam.

When sections were stained with polyvalent rabbit anti-HBs, they were treated before the DAB reaction with HRPO-conjugated swine anti-rabbit antiserum and then with HRPO-conjugated rabbit anti-peroxidase complex (PAP) in order to enhance the specific staining. Background staining with this technique was minimized by initial treatment of the section with normal swine serum.

Specificity. Non-specific staining was minimized by determination of the optimum

Table 1. *Reactivity of mouse monoclonal anti-HBs in the antibody capture radioimmunoassay*

HBsAg positive sera* ...	P1	P2	P3	P4	P5	P6	P7	P8	P9
Clone MHHBS 1	1·01†	1·03	0·94	1·02	1·01	1·04	1·22	1·05	0·77
Clone MHHBS 2	1·32	1·08	1·10	0·98	1·08	1·01	1·27	1·05	0·97

* HBsAg subtype panel; 1st International Workshop, Paris, 1978.

† Expressed as ratios between ^{125}I rabbit anti-HBs binding on solid-phase monoclonal antibody and that on solid-phase polyclonal horse anti-HBs.

dilution for each reagent and subsequent use of the reagent at that dilution. Confirmation of the specificity of staining reactions included the omission of the monoclonal or polyclonal anti-HBV antisera, the substitution of the anti-HBV reagents with either anti-rubella monoclonal antibody or OKT3 (Ortho Laboratories Ltd) and the absence of reactivity with any of the six HBV-negative liver biopsies.

RESULTS

Production of anti-HBV-secreting clones

Anti-HBs

More than 100 anti-HBs clones were derived from 47 selected anti-HBs-secreting hybrid parents generated in three fusions. Selected clones were assayed for their ability in ACRIA to react with a wide range of HBsAg subtypes. Six anti-HBs clones, MHHBS 1–6, were selected on the basis of broad reactivity for HBsAg recognition. For example, the reactivity of the clone MHHBS2 in ACRIA, is directed against a highly conserved epitope and the antibody recognizes all the serotypes P1 to P9 included in the First International Workshop antigen sub-type panel (Table 1), as well as a number of *ay* and *ad* HBsAg-positive sera from geographically widespread sources (data not shown). Ascitic fluids from MHHBS 1–6 were used in subsequent immunohistological studies.

Anti-HBc

Eleven anti-HBc clones were successfully derived from six out of ten anti-HBc-positive hybrids selected from 22 anti-HBc hybrid parents which were generated in a single fusion experiment.

Hybrids secreting anti-HBc were initially detected by testing fusion SNF in ACRIA. This method clearly differentiated antibody-secreting hybrids from those not secreting antibody (Figure 1).

Six clones MHHBC 1–6, one representative of each parent, were selected on the basis of stability of antibody production *in vitro* and propagated as ascitic tumours. They were used in subsequent immunohistological studies. Anti-HBc production *in vitro* was also measured by testing SNF in the competitive RIA. Although all were reactive in ACRIA the level of inhibition was characteristic for all individual clones arising from a single hybrid parent and in many instances would not have been reliably detected by the competitive RIA alone (Table 2).

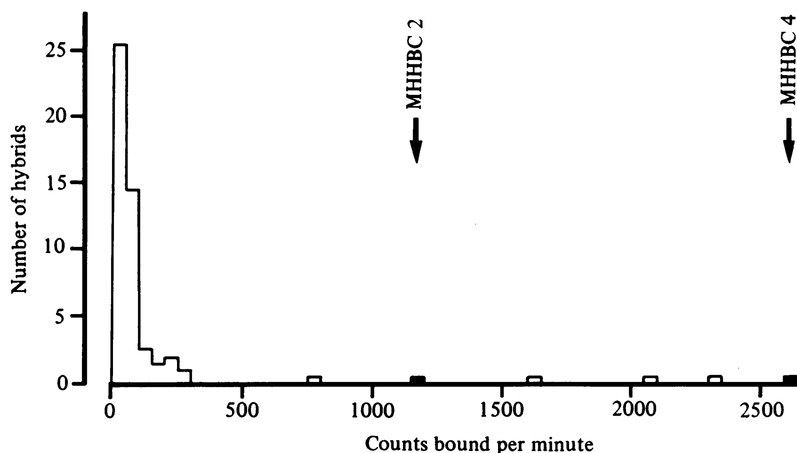

Fig. 1. Distribution of ^{125}I-anti-HBc binding in the antibody capture radioimmunoassay. Supernatent fluid from all 96 wells of a microtitre fusion-master plate were assayed for anti-HBc. Six anti-HBc secreting hybridomas were present on this plate and two (MHHBC 2 and 4) were successfully cloned later.

Table 2. *Comparison of monoclonal anti-HBc reactivity in competition and capture radioimmunoassay*

Anti-HBc (MHHBC) clones	1	2	3	4	5	6
Inhibition in competitive RIA (%)*	77	40	51	56	32	88
Test/negative† ratio in capture RIA	13·8	9·8	8·4	14·4	11·4	4·7

* Negative medium 1145 c.p.m.; strong anti-HBc positive, 12 c.p.m.
† Negative medium, 100 c.p.m.

Immunofluorescent studies and reagent selection

Anti-HBs MHHBS clones

Initially the six fluids were assessed by indirect immunofluorescence on fixed sections. Clones 1 and 2 gave very strong fluorescence; clones 3 and 4 also were reactive but less so and clones 5 and 6 gave no reaction. On this basis clones MHHBS 1 and 2 were further assessed by indirect immunoperoxidase staining on formalin-fixed and wax-embedded liver biopsy material. Both gave strong staining of hepatocytes. It was marginally more intense with clone 2, and this was therefore selected for further investigation.

Anti-HBc MHHBC clones

Initially the six fluids were assessed for reactivity by indirect immunofluorescence on fixed sections. All but MHHBC 5 reacted, although the reaction of clones 1 and 2 was most pronounced. The specificity of this reaction for HBcAg was confirmed by blocking with human anti-HBc (DE52 prepared IgG). Clone MHHBC 1 was also reactive by indirect immunoperoxidase on formalin-fixed and wax-embedded liver and was selected for further investigation.

Indirect immunoperoxidase studies

Specific reactivity for HBsAg was observed as dark brown deposits at the site of the DAB reaction. In the biopsy tissue from the HBsAg-positive patients, the reaction was localized in the cytoplasm of hepatocytes either scattered throughout the lobule or grouped together in clusters (Plate 1). In one instance, HBsAg was demonstrated at the periphery of the liver cell cytoplasm. In all cases the pattern of HBsAg localization was similar when monoclonal anti-HBs was compared in an indirect technique with polyclonal rabbit anti-HBs used in the peroxidase-anti-peroxidase (PAP) technique. However, for any given degree of positive staining of HBsAg, the background staining was always significantly less when the monoclonal rather than the polyclonal anti-HBs reagent was used. No reaction was detected with the liver biopsies from the patients with HBV-negative liver disease, nor in the absence of a specific anti-HBs reagent.

Reactivity for HBcAg was localized mainly within hepatocyte nuclei and occasionally faintly within the cytoplasm of some cells (Plate 2). The reaction was of varying intensity; nucleoli were often seen as discrete nuclear areas which failed to stain for HBcAg. Nuclei containing HBcAg were either scattered throughout the liver lobules or distributed in groups. The pattern of HBcAg reactivity was the same with both the monoclonal and the human polyclonal anti-HBc reagent though, once again, the background staining with the monoclonal reagent was noticeably lower. No staining was seen with the HBV-negative biopsies, in the absence of a specific anti-HBc reagent, or when sections were blocked with human anti-HBc.

DISCUSSION

Selection of antibody-secreting hybrids after a successful fusion will always necessitate a method of testing for antibody which is sensitive yet capable of assaying hundreds of specimens in a short time. The current commercial assay for anti-HBs (AUSAB, Abbot Ltd) is suitable but expensive. Although it is possible to use a solid-phase RIA of this type for detecting anti-HBs production, as shown here, this assay will reveal little about the epitope against which the clone reacts, nor will it differentiate between the classes of immunoglobulins. In this study a solid-phase immunoglobulin-capture RIA was used to provide information on the specificity of the monoclonal anti-HBs. The assay enabled early selection of clones of defined specificity at a stage where only the small quantities of immunoglobulin contained in the SNF from plate tissue-culture wells were available. It is possible to avoid the final step of the assay if endogenously labelled HBsAg is used. Also, if the solid-phase is coated with class-specific anti-mouse Fc the assay becomes a convenient screening method with which to detect clones or hybrid parents secreting anti-HBs of a specific immunoglobulin class (data not shown).

With antigens that are only partially purified, such as HBcAg, capture RIA offers a useful alternative to the conventional indirect-immunoassay using immobilized antigen and labelled anti-mouse-immunoglobulin. In particular, where an antigen is poorly characterized and exists only as an enriched fraction, capture assays may be the only method available. Many anti-HBc-secreting hybrid parents

were detected with this assay and ten were selected for cloning because of a high reactivity. Six yielded monoclonal cultures. As an alternative, the competitive RIA for anti-HBc would not have been entirely satisfactory. The human anti-HBc which was used as the labelled tracer in the competitive RIA recognizes most of the epitopes on the core antigen. As the monoclonal anti-HBc recognizes only a single epitope, the level of inhibition of ^{125}I anti-HBc binding brought about by the monoclonal IgG will reflect the expression of that epitope on the solid-phase HBcAg. If inhibition of label binding is considered to reach a level of significance only at 50% or more, it is not surprising if clones recognizing less common epitopes only react at insignificant levels in the competitive RIA.

In the small number of biopsies examined in this study with monoclonal antibodies to HBcAg and HBsAg there was complete correlation between the virus antigen expression demonstrated with these reagents and that seen with conventional human and rabbit polyclonal antisera. However, although monoclonal antibodies are of the highest specificity, in this lies their susceptibility. Loss of, or major reduction in, reactivity with viral antigens may result from minor antigenic drift. Sometimes a single nucleotide change is sufficient to reduce greatly the reactivity of an antigen with a monoclonal antibody (Webster & Berton, 1981). The anti-HBs clones described here were selected from the large number available on the basis of broad antigen reactivity in ACRIA. This has been confirmed by their reactivity with a wide range of HBsAg positive sera and with the panel of HBsAg seral described by the First International Workshop in 1978. Although MHHBS 2 has also been shown to react in an unbiased manner against very many HBsAg positive sera, until it has been in wide use for a longer period of assessment, these results must be interpreted with caution. The same must apply to the MHHBC clones.

Both the anti-HBs and the anti-HBc monoclonal reagents had an advantage over conventional antisera in that for a given intensity of positive staining the background staining was lower. The increased discrimination with these reagents is a considerable advantage, as in our hands the monoclonal reagents produced staining of the intensity expected for the PAP method but without any increase in background reactivity. An additional advantage is the lack of unwanted specificities, usually the result of the redundant immunoglobulin present in polyvalent sera. From this small series and the data shown here it is not possible to say whether there will always be an advantage with these reagents.

Selected monoclonal reagents have compared favourably with conventional polyclonal antisera in the demonstration of HBV antigens in liver biopsy tissue. It is now possible to have defined high-titre reagents of known specificity which can be made available relatively easily to immunohistologists for comparative studies.

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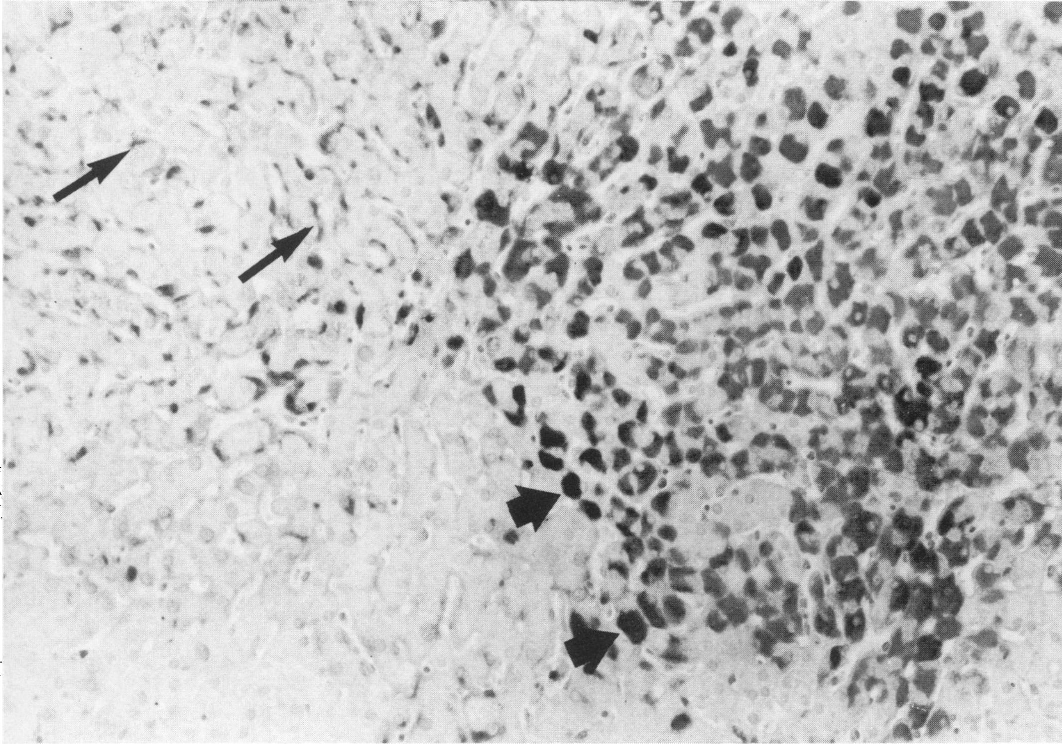
EXPLANATION OF PLATES

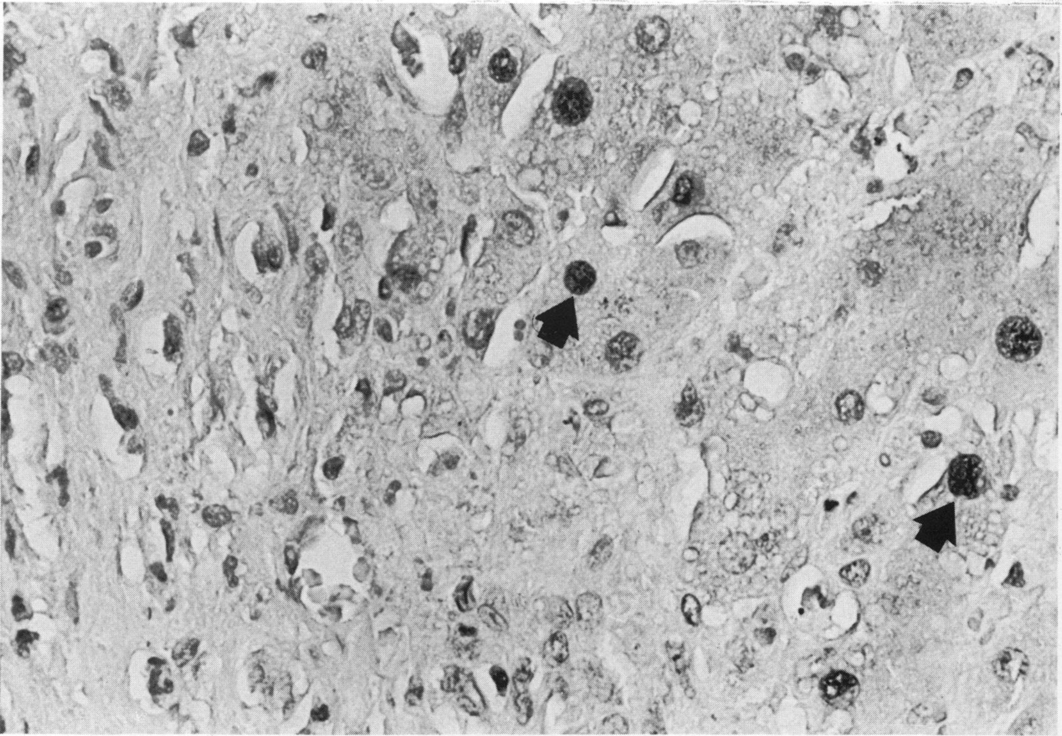
PLATE 1

Liver biopsy from an asymptomatic healthy carrier. Cells containing HBsAg are widespread in the liver lobule. Antigen is present either throughout the entire cytoplasm (thick arrows) or at the periphery of the cells (thin arrows). Staining by indirect immunoperoxidase using monoclonal anti-HBs (Magnification ×280).

PLATE 2

Liver biopsy from a patient with cirrhosis and chronic renal failure. Nuclei containing HBcAg (examples arrowed) are scattered throughout the field. The intensity of the positive reaction varies. Staining by indirect immunoperoxidase using monoclonal anti-HBc (Magnification ×400).





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