

# Detection of Tumour Antigens in Sera of Patients with Hepatocellular Carcinoma Using Monoclonal Antibodies

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Alphafetoprotein (AFP) was first detected in hepatocellular carcinomas (HCC) by using heteroantisera.<sup>1</sup> HCC patients often have an elevated serum AFP level.<sup>2</sup> Similar oncofoetal proteins have also been demonstrated in other neoplasms such as teratocarcinomas.<sup>3</sup> The detection of such antigens in HCC patient sera have been proven to have diagnostic value. Five HCC-specific monoclonal antibodies (mAbs) have been produced from fusions performed using HCC cells as immunizing antigens.<sup>4</sup> The present study was undertaken to assess the reactivities of these characterized mAbs with sera from patients with HCC and other liver diseases.

## MATERIALS AND METHODS

### Monoclonal antibodies

Monoclonal antibodies against human HCC were generated by immunizing BALB/c mice with human hepatoma cell line cells - PLC/PRF/5. Out of 263 mAbs (in the form of culture supernatants) screened against HCC cells, other tumours and normal tissues, 5 had specific reactions with only human HCC cells.<sup>4</sup> These were designated 2G9, 3H11, 3H12, 1C7 and 3H5. 3H11, 3H12, 1C7 and

**SUMMARY** A total of 70 serum samples taken from patients with hepatocellular carcinoma (HCC), acute viral hepatitis and cirrhosis and normal individuals were tested in a binding inhibition immunofluorescence assay using 5 mouse monoclonal antibodies (2G9, 3H11, 3H12, 1C7, 3H5) specific for hepatoma cells. Seven out of the 30 HCC sera (23.3%) inhibited the binding of one of these antibodies, 3H11. This detection of antigen 3H11 or antigens of similar structure in HCC sera was significantly more frequent than in control sera (1/40 = 2.5%) (Fisher's exact test,  $p = 0.009$ ;  $df = 1$ , relative risk calculated by the odds ratio in a  $2 \times 2$  table = 12.0). The presence of this antigen was unrelated to the hepatitis B surface antigen and alphafetoprotein status. Thus it would be of value particularly for the detection of hepatitis B negative or alphafetoprotein negative liver cancers.

3H5 gave significant immunofluorescent binding up to a dilution of 1:125; 2G9 gave up to 1:25. 3H11, 3H12 and 1C7 consistently exhibited plasma membrane or plasma membrane plus cytoplasmic staining. 2G9 and 3H5 reacted with cytoplasmic antigens. These 5 antibodies demonstrated similar immunocytochemical reactivity patterns with other human HCC cells, Hep G2 and Hep 3B. Screening on hepatoma cells was carried out by indirect immunofluorescence. 2G9, 3H11 and 3H12 were identified as IgG 2a, 1C7 as IgG 2b and 3H5 as IgG 3 by double immunodiffusion.

### Preparation of cell suspensions and monolayers

Target cells (PLC/PRF/5) were grown in MEM medium and cell pre-

parations obtained by trypsinization. These were prepared as live, unfixed cells to detect membrane staining. Cells were also cultured in monolayers on multi-chamber culture glass slides (Lab-Tek, Miles Labs, USA) for a minimum of 2 days at 37° C. These were fixed in 95 percent methanol.

### Storage of sera

Sera from individuals in whom the diagnoses were pathologically

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proven, were frozen in aliquots and tested when required and these included 30 hepatocellular carcinoma (HCC), 20 acute viral hepatitis (AVH), 10 cirrhotic patients and 10 normal subjects.

#### Immunofluorescence assays

The positive assay system for detecting mAb reaction with PLC/PRF/5 cells was by indirect immunofluorescence using fluorescein isothiocyanate (FITC)-labelled rabbit anti-mouse Igs (Dakopatts, Glostrup, Denmark). Serum samples taken from patients with HCC, patients with other liver diseases and from healthy volunteers were tested in a binding inhibition immunofluorescence assay. Test serum diluted 1:16 was mixed with each mAb and the mixtures incubated with target cells for 30 minutes at room temperature. The cells were washed and the FITC-labelled rabbit anti-mouse Igs (Dakopatts, Glostrup, Denmark) added and incubated for 30 minutes at room temperature. Staining reactions were evaluated by fluorescence microscopy (Carl Zeiss Universal microscope III RS, West Germany). Positive and negative controls were used in every assay. The sera were tested for their ability to inhibit the binding of mAb to the target liver cancer cells. Inhibition of any mAb reaction by the serum was considered positive when the staining reaction was reduced by half or more as compared to the mAb staining without the serum. After absorption, there was a complete reversion from positive to negative fluorescence.

#### Tests for hepatitis A and B markers and AFP

Sera were tested using counter immunoelectrophoresis,<sup>5</sup> reverse passive hemagglutination (Abbot laboratory, USA) and radioimmunoassays (Abbot lab, USA). Hepatitis markers tested included HBsAg, antibody to HBsAg, antibody to hepatitis B core antigen and IgM

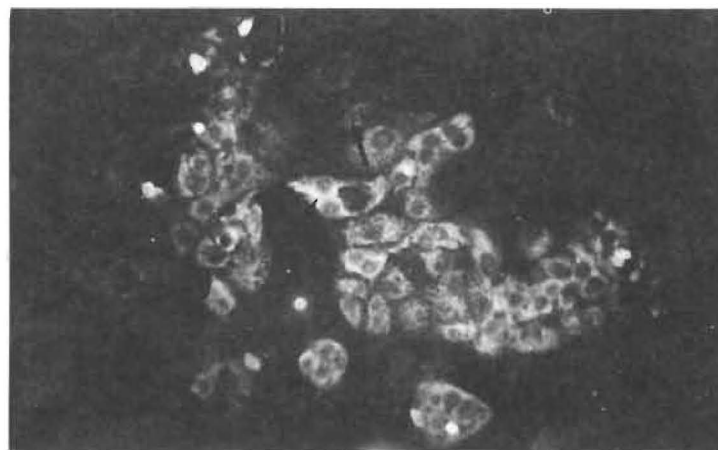
and IgG antibodies to hepatitis A virus. Blocking immunofluorescence tests were carried out on PLC/PRF/5 cells to confirm that mAbs were not detecting AFP or HBsAg.

#### RESULTS

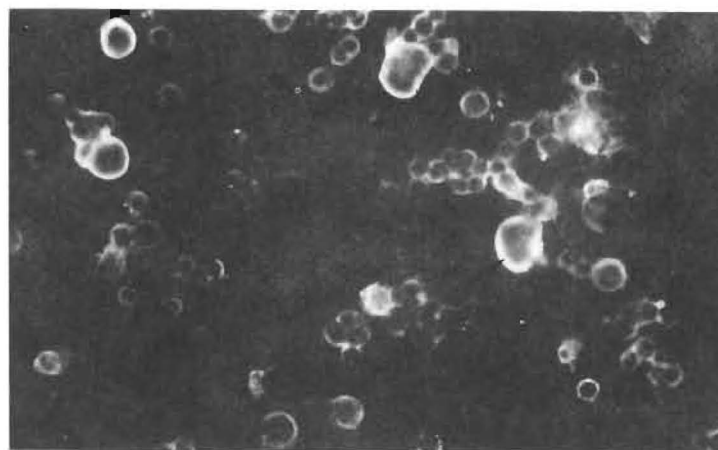
The reaction patterns of 2G9 and 3H11 on PLC/PRF/5 cells detected by immunofluorescence are shown in Figures 1 and 2. The cellular localization of 3H5 is similar to that

of 2G9; that of 3H12 and 1C7 is also similar to that of 3H11. Sera from some patients with liver diseases could block the reaction between some of the mAbs and PLC/PRF/5 cells. The frequency of such blocking or inhibition activity in the various groups of liver disease sera is summarized in Table 1.

The reaction of mAbs 1C7 and 3H5 could not be blocked by any of the sera tested and none of the 10



**Fig. 1** Human hepatoma cells (PLC/PRF/5) show a granular fluorescent pattern localized in the cell cytoplasm when stained with mAb 2G9 (arrows). No counterstain. X200.



**Fig. 2** Staining with 3H11 results in linear fluorescence of the plasma membrane in PLC/PRF/5 cells (arrows). Cytoplasmic staining is also displayed in some cells. No counterstain. X80.

normal sera showed any blocking reactivity to any of the 5 mAbs (Table 1). The reaction of mAb 3H12 was blocked by 3/30 (10%) sera from HCC patients and 5/20 (25%) sera from AVH patients but none of the 10 sera from patients with cirrhosis nor the 10 normal sera. The frequency of blocking of mAb 3H12 by sera from AVH patients was significantly higher than all other sera combined ( $3/50 = 6\%$ ;  $p = 0.03$ , Fisher's exact test). The reaction of mAb 2G9 was also blocked by a small proportion of sera from patients with AVH and cirrhosis but was not statistically significant. The reaction between mAb 3H11 and PLC/PRF/5 cells was blocked by 7 (23.3%) of 30 HCC sera compared to only 1 (2.5%) of 40 non HCC sera ( $p = 0.0009$ ;  $RR = 12.0$ ). This one positive non-HCC serum was taken from a patient with cirrhosis, which can be a pre-malignant condition. The frequency of inhibition activity of 3H11 by HCC sera was also significant when compared to that of sera from other liver diseases ( $p = 0.024$ ).

There was no correlation between HBsAg nor AFP levels and the mAb reactivity inhibition by any of these sera.

## DISCUSSION

Antigens may be released by certain tumour cells and could be detected in the sera of diseased patients.<sup>6</sup> A previous report stated that 24 out of 33 sera from colorectal carcinoma patients inhibited mAb binding to target cells by more than 19 percent in a inhibition radioimmunoassay.<sup>7</sup> Sera from 6 patients with other bowel diseases including 2 with multiple polyps were also tested. Serum from a patient with colitis inhibited binding by more than 10 percent. Another report made on melanoma patient sera did not include other related disease sera.<sup>8</sup> None of 87 melanoma sera inhibited significantly the reactivity of anti-melanoma mAbs with target cells in

**Table 1.** Number of patient sera reactive in immunofluorescence inhibition assay

Serum donor Group	Total No Each Group	Monoclonal antibodies				
		2G9 (%)	3H11 (%)	3H12 (%)	1C7 (%)	3H5 (%)
HCC*	30	0 (0)	7 (23.3)	3 (10)	0 (0)	0 (0)
AVH**	20	2 (10)	0 (0)	5 (25)	0 (0)	0 (0)
Cirrhosis	10	1 (10)	1 (10)	0 (0)	0 (0)	0 (0)
Normal	10	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

\*Hepatocellular carcinoma  
\*\*Acute Viral Hepatitis

an indirect rosette assay. The discrepancies of the findings by the two groups of investigators may be mainly due to different assay systems.

In our study, an inhibition assay was conducted to test the ability of HCC and other sera to block the immunofluorescence reactivities of mAbs on liver cancer target cells. In this application, this assay was sensitive enough to demonstrate the inhibition of binding of 3H11 in 23.3 percent of HCC sera. Some of these sera contained antigens which reacted with the mAbs. The antigens presumably shared epitopes with those to which the mAbs react on the target liver cancer cells. These common antigens detected in human sera are probably not related to hepatitis B and A viruses since there is no correlation between such antigens and hepatitis B and A viral markers. They are unlikely to be related to HBV<sub>2</sub> virus<sup>9</sup> because the surface antigen of hepatitis B virus and HBV<sub>2</sub> share some epitopes. The question arises as to whether these antigens could have been induced by another virus and if so, may raise the possibility of another virus coexisting with hepatitis B or A virus (eg. non A, non B).

No significant correlation is evident in those HCC patients who were positive for the serum antigens with respect to clinical presentations

and survival. A 30 year old female who had previously been on oral contraceptives for 10 years and developed liver cancer was tested positive.

The serum antigen as detected by 3H11 was present more frequently in HCC than other related sera. The serum of one non-HCC patients with cirrhosis blocked 3H11 reactivity. We are following this patient up to see if he develops HCC. This was not blocked by HBsAg or AFP so perhaps a combination of 3H11 with AFP and HBsAg can cover a wider range of liver cancer detection.

## ACKNOWLEDGEMENTS

We thank Dr. J. Alexander, National Institute of Virology, South Africa, Drs. B. Knowles and D. Aden, Wistar Institute, Philadelphia, PA for their gifts of the PLC/PRF/5, Hep G2 and Hep 3B cell lines. This study was supported by grants from Research Development Assistance Scheme, Ministry of Trade and Industry, Lee Foundation, Shaw Foundation, Singapore Turf Club and W.H.O. Geneva.

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