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ORIGINAL RESEARCH

Preparation and application of monoclonal antibodies against hepatitis C virus nonstructural proteins

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Abstract

AIM: To prepare hybridoma cell lines that secrete monoclonal antibodies against hepatitis C virus (HCV) recombinant proteins NS3 and NS5 and to evaluate their use in the study of HCV NS3 and NS5 antigen distribution in human liver tissue.

METHODS: Hybridoma cell lines were generated using spleen cells from BALB/C mice immunized with recombinant NS3 and NS5 proteins, following conventional protocols. Antibody-secreting cells were screened by solid phase ELISA and cloned by limited dilution. The specificity of the monoclonal antibodies was determined by testing hybridoma culture supernatants by Western blots of *E. coli* expressing the recombinant HCV proteins and ELISA with HCV core and hepatitis B virus (HBV) antigens. The monoclonal antibodies were employed in immunohistochemistry studies to determine the distribution of HCV NS5 and NS3 antigens in 51 paraffin embedded human liver tissue samples.

RESULTS: Eight hybridoma cell lines secreting monoclonal antibodies against HCV NS3 and NS5 proteins were generated and named 2B6, 2F3, 3D8, 3D9, 8B2, 6F11, 4C6 and 7D9. Only one of them, 2B6 (secreting antibodies against NS3 protein), cross-reacted with the C7 polypeptide, a different recombinant NS3 polypeptide. The rest of the cell lines showed no cross-reactivity with HCV core or HBV antigens. In addition, monoclonal antibodies against NS3 antigens did not cross-react with NS5 antigens, and vice versa. In immunohistochemistry studies, these monoclonal antibodies did not detect HCV antigens in specimens from patients infected only with HBV (n = 20). In HCV-infected specimens (n = 31), the rates of positive detection of NS3 and NS5 antigens were 51.6% (16/31) and 54.9% (17/31), respectively. Six of these 31 specimens were from patients infected only with HCV and half of them were positive for HCV NS3 and NS5 antigens. In specimens from patients co-infected with HBV and HCV (n = 25), the rates of NS3 and NS5 antigen positive detection were 52% (13/25) and 56% (14/25), respectively, which are similar to those obtained in samples from patients infected only with HCV. In specimens from chronic active cirrhosis patients, the rates of HCV NS3 and NS5 antigen detection were 70.6% (12/17) and 76.5% (13/17), respectively.

CONCLUSION: We successfully prepared monoclonal antibodies that are specific against recombinant HCV NS3 and NS5 proteins and could be useful for clinical immunohistochemistry diagnosis.

Key words: Hepatitis C virus; Antibodies; Monoclonal; Viral proteins; Antigens; Viral

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INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of non-A, non-B hepatitis that is associated with the development of cirrhosis and hepatocellular carcinoma (HCC)[1]. Although the mechanism of liver injury by HCV infection is still unknown, replication of HCV plays a key role. The diagnosis of HCV infection and the assessment of the effectiveness of interferon (IFN) therapy for its treatment are mainly performed by serological assays. These assays include the detection of anti-HCV IgG antibodies in serum and of HCV RNA by RT-PCR, which can only reflect the presence of actively replicating virus[2]. Tsutsumi *et al*[3] (1994) reported that although HCV RNA in serum was negative after IFN therapy, HCV NS5 antigen was still present in liver cells from IFN-treated patients.

NS3 and *NS5* are important genes involved in virus replication and assembly. *NS3* encodes a putative helicase and *NS5* encodes a putative RNA-dependent RNA polymerase[4]. Monoclonal antibodies against these two proteins would be useful for the determination of HCV state

Table 1 Immunochemistry analysis of liver disease samples (n = 51)

Diagnosis	HCV-infected					Co-infected with HCV and HBV					HBV-infected				
	No.	HBsAg	HBcAg	NS3Ag	NS5Ag	No.	HBsAg	HBcAg	NS3Ag	NS5Ag	No.	HBsAg	HBcAg	NS3Ag	NS5Ag
CAC	3	0	0	2	2	14	13	8	10	11	9	9	6	0	0
SAFH	1	0	0	0	0	1	0	0	0	0	2	1	1	0	0
CFH	2	0	0	1	1	5	4	3	2	2	5	4	2	0	0
HCC	0	0	0	0	0	5	3	1	1	1	4	3	1	0	0
Total	6	0	0	3	3	25	20	12	13	14	20	17	10	0	0

HCV: Hepatitis C virus; HBV: Hepatitis B virus; CAC: Chronic active cirrhosis; SAFH: Sub-acute fulminant hepatitis; CFH: Chronic fulminant hepatitis; HCC: Hepatocellular carcinoma.

Table 2 Anti-NS3 monoclonal antibody cross-reactivity with other antigens

Monoclonal Antibodie	CP9	CP10	C11	C7	HBsAg	NS3Ag
2B6	0.075	0.081	0.124	1.591	0.079	2.378
2F3	0.074	0.085	0.096	0.089	0.161	2.233
3D8	0.091	0.082	0.086	0.078	0.136	2.139
3D9	0.076	0.081	0.081	0.079	0.113	2.350

Table 3 Anti-NS3 epitope analysis

Monoclonal Antibodie	Inhibition rate (%)			
	2B6	2F3	3D8	3D9
2B6	100.0	0	0	0
2F3	0	100.0	97.9	101.9
3D8	30.0	103.6	100.0	110.0
3D9	36.0	93.5	93.0	0

in liver cells and for immunochemistry studies on type C hepatitis pathogenesis. In addition, they will greatly help in the development of methods to determine the success of IFN therapy. Therefore, we generated monoclonal antibodies against recombinant HCV NS3 and NS5 proteins and tested them in immunohistochemistry studies using paraffin embedded liver tissue sections from HCV-positive patients.

MATERIALS AND METHODS

Patients

Fifty-one autopsy specimens of human liver from patients with type C and type B hepatitis were obtained. Among them, 31 were HCV-positive and 20 were hepatitis B virus-positive (HBV-positive). Twenty-six of these cases had active cirrhosis, four showed sub-acute fulminant hepatitis, 12 had chronic fulminant hepatitis and nine showed hepatocarcinoma (Table 1). All specimens were stained with hematoxylin-eosin, HBV antigens (HBsAg and HBcAg), and HCV antigens (NS3Ag and NS5Ag), separately. Three liver biopsies from non-liver disease patients served as controls. In order to confirm specificity we replaced first antibody with mouse serum, human serum, or PBS in control assays.

Monoclonal antibody preparation

BALB/c mice were immunized 3 times at a 2-wk interval with recombinant NS3 (30 kDa) and NS5 (27 kDa) polypeptides expressed in *E. coli*. Three days after the last immunization, spleen cells were obtained and fused with Sp2/0 myeloma cells, following conventional methods for the generation of monoclonal antibodies (McAbs). Antibody-secreting cells were screened by solid phase ELISA and cloned by limited dilution[5].

Monoclonal antibody characterization

The immunoglobulin subclasses of McAbs were determined following conventional methods. The specificity of McAbs was established by testing cross-reactivity of culture supernatants from hybridoma cells in Western blots of membranes containing HBsAg, CP9, CP10, C7, and C11 antigens[6]. McAb epitopes were analyzed by an inhibition assay.

RESULTS

The fusion rates after cell fusion were 100%; 30%-40% of hybridoma cells were antibody-secreting. Four hybridoma cell lines secreting antibodies against NS3Ag (2B6, 2F3, 3D8, and 3D9) and four

Table 4 Anti-NS5 monoclonal antibody cross-reactivity with other antigens

Monoclonal Antibodie	CP9	CP10	C11	C7	HBsAg	NS3Ag	NS5Ag
8B2	0.065	0.054	0.078	0.088	0.098	0.098	2.325
6F11	0.055	0.053	0.076	0.078	0.097	0.088	1.987
4C6	0.065	0.067	0.078	0.090	0.085	0.102	2.165
7D9	0.070	0.072	0.085	0.092	0.070	0.103	2.100

hybridoma cell lines secreting antibodies against NS5Ag (8B2, 6F11, 4C6, and 7D9) were cloned and further characterized. Hybridoma cell line 2B6 was the only cell line that cross-reacted with the C7 polypeptide, a different recombinant NS3 polypeptide, the rest of the hybridoma cell lines showed no cross-reactivity with other antigens (Table 2).

Anti-NS3Ag McAbs reacted against two different NS3 epitopes (Table 3); anti-NS5Ag McAbs reacted against the same NS5 epitope (Table 4).

The McAbs generated against HCV NS3Ag and NS5Ag were tested by Western blot using membranes prepared by SDS-PAGE of *E. coli* expressing recombinant NS3, recombinant NS5, and non-recombinant plasmid. A single band with a molecular weight of 30 kDa was observed for recombinant NS3 plasmid, whereas two bands with molecular weights of 27 kDa and 23.5 kDa were observed in the *E. coli* expressing recombinant NS5 plasmid.

Using these McAbs in immunohistochemistry studies, HCV NS3Ag and NS5Ag were visualized only in liver sections from patients positive for HCV but not in sections from patients with only HBV infection (Table 1). We identified three types of staining pattern in hepatocytes: Diffuse, clustered, and patchy. In samples from HCV-infected patients, the rates of positive HCV NS3Ag and NS5Ag were 51.6% (16/31) and 54.9% (17/31), respectively. In samples from patients infected only with HCV, 50% (3/6) were positive for NS3Ag and NS5Ag. Out of the 25 specimens from patients co-infected with HCV and HBV, 13 specimens were positive for NS3Ag and 14 specimens were positive for NS5Ag. The rate of samples positive for HCV antigens was not significantly different between these two groups ($P > 0.1$).

DISCUSSION

NS3 and *NS5* are important *HCV* genes encoding non-structural proteins of the virus (a putative helicase and an RNA-dependent RNA polymerase, respectively)[4]. Since HCV is a single positive stranded RNA virus distantly related to flaviviruses[7], a helicase and an RNA-dependent RNA polymerase may be necessary for HCV replication. The presence of HCV NS3Ag and NS5Ag in liver tissue from patients may reflect the replication state of the virus. In this study, we used recombinant NS3 and NS5 polypeptides (with molecular weights of 30 kDa and 27 kDa, respectively) expressed in *E. coli* to immunize mice and generate several strains of McAb-secreting hybridoma cells. These McAbs were proved stable by *in vitro* transmission for three months.

By Western blot analysis, we demonstrated that the anti-HCV NS3 and anti-HCV NS5 McAbs were specific against the antigens they were raised against. The detection of two bands with molecular weights of 27 kDa and 23.5 kDa in Western blots of NS5-expressing bacteria may indicate the presence of a partially degraded polypeptide in addition to the full-length one.

Epitope analysis of anti-NS3 McAbs identified at least two different epitopes for this molecule, one of them being the same as for C7, which is a different NS3 recombinant polypeptide.

By immunohistochemistry studies, we observed nuclear staining of NS5Ag in one case. As this observation differs from the results of

Tsutsumi *et al*[3], further analyses are needed. The rate of samples positive for HCV antigen was similar between samples from patients infected only with HCV and samples from patients co-infected with HCV and HBV, indicating that the McAbs we generated are specific to HCV antigens. The rate of samples positive for HBV antigens is higher than that of HCV antigens. This observation may be explained by the fact that HBV antigens (HBsAg and HBcAg) reflect the presence of the virus, whereas HCV antigens (NS3Ag and NS5Ag) reflect the replication state of the virus.

In conclusion, we have generated McAbs that are specific against HCV NS3Ag and NS5Ag and have good prospects for application in HCV immunochemistry studies. As the presence of these two antigens reflects the replication state of HCV, they may become useful markers for HCV-infection diagnosis and treatment.

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